The Role of the Gene Expression in the Lateral Hypothalamus on the Development of Allodynia After Cisplatin Treatment

by

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Dedication

This dissertation is dedicated to my mother Elsy Young Wagner and my father James Dale Wagner and my older brother James Jason Wagner. They have provided me with love and support throughout my entire life, but especially during my time spent at the University of Michigan completing this dissertation. They have also instilled in me the perseverance, determination, and strength that have helped me to become the person that I am.

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ABSTRACT

Advances in cancer treatment have led to an increase in cancer survivorship as the number of individuals living beyond cancer diagnosis and treatment rises each year. Yet, as this number rises, so does the number of people who live with the side-effects of cancer treatment. Chemotherapy-induced peripheral neuropathy (CIPN) is a common side-effect of neurotoxic chemotherapeutic drugs that is characterized by sensations of numbness and tingling that begin in the distal extremities and can develop into chronic neuropathic pain. There are no recommended preventative measures for CIPN and the only recommended treatment is the serotonin-norepinephrine reuptake inhibitor, duloxetine, which does not offer relief to all patients. The lateral hypothalamus (LH) is part of a descending system that modulates pain in the spinal cord dorsal horn. The chemotherapeutic drug cisplatin accumulates in the dorsal root ganglion of the primary afferent neuron and promotes abnormal pain signaling that may disrupt the genome of LH cells, via the spinohypothalamic ascending tract, contributing to the development of painful CIPN.

This dissertation study investigated the role of the LH in the development of cisplatin-related mechanical allodynia, a sign of neuropathic pain. The goal was to detect changes in LH-derived gene expression and examine the association of these changes with development and persistence of mechanical allodynia in different strains of mice. Results of nocifensive behavioral von Frey testing have shown that different strains of mice (C57BL/6J, BALB/cJ, DBA/2J, A/J, FVB/NJ, and CD-1) develop allodynia at different rates post cisplatin treatment. Based on these results, the two strains that represented the highest (C57BL/6J) and lowest (A/J)

responders to allodynia development were selected for further gene expression and protein analysis.

Analysis of microarray data from the LH transcriptome of C57BL/6J and A/J mice showed very few gene expression changes within each strain after cisplatin treatment, but we discovered 1311 differentially expressed genes (DEGs) between the strains **prior to** treatment. Using Ingenuity Pathway Analysis (IPA) to characterize these DEGs, we discovered that the top biological pathways affected by the DEGs included "Synaptic Long-Term Potentiation" and "Neuropathic Pain Signaling in Dorsal Horn Neurons." Using IPA, we identified nine genes with documented function in pain development for further target gene expression validation with quantitative real-time polymerase chain reaction (qPCR). The results of the qPCR were used to verify the gene expression direction in seven of the DEGs. Western blot confirmed two of the genes were present at the protein level.

The results of this pre-clinical study are suggestive that the gene expression profile **prior to** cisplatin treatment may predispose the development of painful CIPN in mice. These pre-clinical findings may ultimately guide precision health by using gene expression profiles to predict patients at risk for painful CIPN and identify patients who will benefit from preventative measures.

CHAPTER I

INTRODUCTION

Advances in early detection and treatment of cancer have led to almost 14.5 million cancer survivors in the United States alone (Siegel et al., 2012). An increasing amount of research is being devoted to the prevention and treatment of unwanted side effects of cancer treatment. Chemotherapy-induced peripheral neuropathy (CIPN) occurs in up to 68% of individuals who receive neurotoxic chemotherapy (e.g. platinums, vinca alkaloids, bortezomib, and taxanes; Argyriou, Bruna, Marmiroli & Cavaletti, 2012; Cavaletti et al., 2013; Hershman et al., 2014; Kautio, Haanpaa, Kautiainen, Kalso, & Saarto, 2011; Mols, Beijers, Vreugdenhil & van de Poll-Franse, 2014, Seretny et al., 2014). CIPN generally manifests with symptoms of pain, burning, numbness, and tingling in the extremities (Saif & Reardon, 2005; Smith et al., 2014). CIPN is reported as painful in 40 – 58% of patients (Kautio, et al., 2011; Loprinzi et al., 2011). It is refractory to traditional treatments and can lead to dose reduction, delay, or even early termination of a potentially successful chemotherapy treatment. After completion of chemotherapy, patients may experience a worsening of neuropathy that can last from several months to years (coasting; Argyriou et al., 2012; Miltenburg & Boogerd, 2014). The pain experienced by those with CIPN can be seriously debilitating, causing functional status impairment, decreased workplace productivity, and an overall decrease in quality of life (Travis et al., 2014). Despite the known negative effects of CIPN on quality of life, few treatments and no preventative measures exist (Hershman et al., 2014).

Statement of the Problem

As the prevalence of cancer increases, so does the incidence of CIPN, a common adverse effect of many chemotherapeutic agents used to treat cancer. CIPN causes severe pain, is refractory to traditional pharmacological treatments and can lead to dose reduction, delay, or even early termination of a potentially successful treatment. CIPN is also associated with increased utilization of health care leading to increased health care costs. It has been reported that in the US, chemotherapy patients that develop CIPN have average healthcare costs that are $17,344 more than those of chemotherapy patients who do not develop CIPN (Pike et al., 2012). In spite of tremendous research efforts, there has been little progress in prevention and treatment for CIPN.

About 60% of patients receiving the platinum-based chemotherapeutic agent, cisplatin, show evidence of peripheral nerve damage (Argyriou et al., 2012), and 20% cannot complete a full course of therapy due to CIPN (McDonald et al., 2005). The severity of CIPN is related to platinum compound accumulations in the dorsal root ganglia (DRG; Argyriou et al., 2014; Dzagnidze et al., 2007; Gregg et al., 1992) that lead to changes in the excitability of neurons in the spinal cord dorsal horn (Scott et al, 1995) through a process called central sensitization. The development of central sensitization produces pain hypersensitivity via a long-term shift in neuron sensitization from high threshold to low threshold (Latremoliere & Woolf, 2009). This shift can alter DNA and the sensory responses provoked by normal input, making normally innocuous sensations painful (Latremoliere & Woolf, 2009), a phenomenon known as allodynia.

The lateral hypothalamus (LH) is part of the descending brain system that modulates pain in the spinal cord dorsal horn (Holden et al., 2014). The LH system is activated by a pain-provoking (nociceptive) stimulus, which causes release of inhibitory neurotransmitters in the dorsal horn that attenuate or block incoming nociceptive action potentials. The central sensitization that develops post cisplatin administration leads to increased nociceptive input into the spinal cord, increasing the nociceptive input to the LH and making the LH vulnerable to genetic change. There exists a gap in the scientific knowledge regarding the relationship between gene expression in the LH and cisplatin administration and how this relationship contributes to the development of allodynia.

Purpose

This dissertation study examined the role of the LH in the development of cisplatin-related mechanical allodynia. The goal was to detect gene expression changes in the LH and examine the association of these changes with development and persistence of mechanical allodynia in different strains of mice. Beginning with six strains of mice (C57BL/6J, BALB/cJ, DBA/2J, A/J, FVB/NJ, and CD-1) to represent the heterogeneity seen in clinical practice, nocifensive behavioral von Frey testing was performed to assess the development of mechanical allodynia. Based on these results, the two strains that represent the highest and lowest responders to allodynia development were then selected for further gene expression and protein analysis. The aim of this study was to determine key genes that may provide at least part of the answer as to why some individuals develop allodynia post cisplatin treatment and others do not.

Specific Aims

The following describes the specific aims and hypotheses that guided the research strategy of the study using various strains of female mice (n = 6-8 per treatment group) given a dose of cisplatin (4 mg/kg twice weekly), while control groups received saline or remained naïve to treatment for four weeks.

Aim 1: Test the role of cisplatin in development of mechanical allodynia as measured by decreased paw withdrawal threshold to grams of pressure on the hind paw (von Frey filaments). We predict that cisplatin will significantly decrease paw withdrawal threshold compared to control mice. We also predict that each strain of mice will develop different degrees of mechanical allodynia as compared to control.

Aim 2: To use both genome-wide expression analysis (microarray) and pathway analysis (Ingenuity Pathway Analysis; IPA) to determine gene expression and compare the relationships between differentially expressed genes in the two strains of mice, that represent the highest and lowest responders to allodynia development, before and after cisplatin treatment. We hypothesize that expression differences will be present in genes associated with the development of CIPN.

Aim 3: To use quantitative real-time polymerase chain reaction (qPCR) to verify gene expression changes and Western blot to confirm the presence of protein translation from the differentially expressed genes. We predict that gene expression changes detected in aim 2 will be verified by both qPCR and Western blot.

Approach

The experience of pain is subjective and highly complex, and the degree of pain that an individual will experience is not necessarily related to the amount of nociceptive input (Bingel & Tracey, 2008). The perception of pain is the result of the processing of nociceptive information and is subject to significant modulations (pro-nociceptive and anti-nociceptive) that can be accomplished through spinal cord reflexes or supra-spinal cognitive factors (Bingel & Tracey, 2008). Descending pain modulation from the cortical areas to the brainstem and then the spinal cord contributes to the endogenous modulation of pain. The research question of this dissertation was guided by the modulation of pain.

Ascending Pathways. Briefly, a noxious stimulus travels via primary afferent nociceptors to the spinal cord dorsal horn. In the dorsal horn, the primary afferent synapses with a second order neuron. The axons of the second order neurons travel via the ascending sensory system to the brain stem, forebrain, and then to the cerebral cortex. The ascending sensory pathways are classified according to anatomical location. The major ascending tracts are the spinothalamic, spinoreticular, spinomesencephalic, spinoparabracial, spinohypothalamic, spinocervical, and postsynaptic pathway of the spinal column (Almeida, Roizenblatt, & Tufik, 2004). Each of these tracts carries the nociceptive impulse from the spinal cord to the brain. The research of this dissertation is concerned with the spinohypothalamic tract (Burstein, Cliffer, & Giesler, 1990). A further discussion of the ascending tracts appears in chapter II.

Descending Pathways. Descending modulation of pain begins in the brain. Via a number of different synapses within areas of the forebrain and brainstem, the impulse from the stimulus returns to the spinal cord, where it is either potentiated (descending facilitation) or suppressed (descending inhibition; Millan, 2002). The LH is part of a descending system that modulates pain at the level of the spinal cord dorsal horn. The connection from the LH to the spinal cord can be either direct (Haghparast, Shafiei, Alizadeh, Ezzatpanah, & Haghparast, 2017, van den Pol, 1999, Wardach, Wagner, Jeong, & Holden, 2016) or indirect via connection in the periaqueductal grey, nucleus raphe magnus, and A7 catecholamine cell group (Aimone & Gebhart, 1987; Aimone, Bauer, & Gebhart, 1988; Ezzatpanah, Babapour, Sadeghi, & Haghparast, 2015, Franco and Prado, 1996; Holden & Naleway, 2001; Holden, Van Poppel, & Thomas, 2002; Holden & Pizzi, 2008; Holden, Pizzi, & Jeong, 2009; Holden, Wagner & Reeves, 2018; Leite-Almeida, Valle-Fernandes, & Almeida 2006; Safari, Haghparast, & Semnanian, 2009; Sim and Joseph, 1992; Wagner, Banerjee, Jeong, & Holden, 2016). Descending modulation from the LH is also further discussed in chapter II.

Animal Model. Due to the exploratory nature of this study, as well as the need for brain tissue from the LH, this research used an animal model of cisplatin-induced allodynia, starting out with five inbred (C57BL/6J, BALB/cJ, DBA/2J, A/J, FVB/NJ; Jackson Laboratories) and one outbred (CD1; Charles River Laboratories) strains of mice. Over 95% of the mouse genome is similar to that of humans (Hardouin & Nagy, 2000), making research with mice applicable to human disease. Each mouse strain was chosen based on previous work showing the differences across mouse strains in response to treatment with the chemotherapeutic taxane paclitaxel (Smith, Crager, & Mogil, 2004). The chosen strains include mice that have been sibling mated for at least 20 generations, making them essentially genetically identical (Smith et al., 2004). The outbred strain contains a larger degree of genetic heterogeneity and was bred for at least four generations (Chia, Achilli, Festing, & Fisher, 2005, Marmiroli et al, 2017)**.**

C57BL/6J. This inbred strain is used as the reference for nocifensive behavior. This general-purpose strain is one of the most commonly used strains for the production of transgenic and knockout animals (Currer et al., 2009). Most pain studies conducted in mice use the C57BL strain, therefore comparisons of our results with those of other laboratories will be easier to facilitate.

BALB/cJ. This inbred strain was selected based on its use in chemotherapy-induced pain and neuropathy studies in the literature (Benbow, et al., 2016, Toyama, Shimoyama, & Shimoyama, 2017; Podratz et al., 2016).

DBA/2J. A comparison between strain sensitivities has determined that several of inbred mouse strains show variability in their sensitivity to pain, with the DBA/2J strain being highly sensitive to pain (Mogil et al., 1999a,b; Smith et al., 2004). We use the DBA/2J strain to compare the nocifensive behavioral responses to persistent pain between a highly sensitive strain and a markedly less sensitive strain (see A/J below).

A/J. Previous reports have determined that the inbred A/J mice appear to be less sensitive to nociceptive stimulation (Mogil, et al. 1999a,b). Therefore, it would be of merit to investigate the decreased pain sensitivity found in the A/J strain in addition to comparing the behavioral responses between A/J mice and other strains of mice.

FVB/NJ. The FVB/NJ inbred strain is sensitive to nociceptive stimulation (Marmiroli et al., 2017; Yowtak, et al., 2011). This strain is also widely used for general purpose studies (Currer et al., 2009).

CD-1. This outbred strain of mouse was used to compare the results of inbred strains to those of an outbred strain. CD-1 mice were also used in pilot studies investigating allodynia produced by the chemotherapeutic drug, paclitaxel (Smith et al., 2004).

Results

In Chapter II, I detail a more in-depth description of the processing of nociceptive information, pathophysiology, and symptoms of cisplatin induced CIPN. Chapter III is presented in manuscript form as it is in preparation for publication. Due to a lack of knowledge in nursing about the molecular methodologies and difficulties in performing these tests, I explain the methods used to perform my research as well as how I was able to troubleshoot the difficulties I encountered during my experiments. Chapter III is meant to serve as a primer of genetic testing from the viewpoint of performing the experiments as well as a methods chapter. Chapter IV presents the results of the Specific Aims in manuscript format. The suggested cisplatin dose produced different degrees of mechanical allodynia in each mouse strain. However, when trying to repeat the results with the strains that developed the most (C57BL/6J) and least (A/J) paw withdrawal percentages from baseline, the A/J mice had to be euthanized after one week of treatment due to severe weight loss (Aim 1). Using microarray as the method of genome-wide expression analysis in the LH, we found very few gene expression changes within a strain after treatment, but hundreds of changes between the strains at baseline, especially between the C57BL/6J and A/J (Aim 2). Further pathway analysis (IPA) led to the identification of many differentially expressed genes with a role in the development of neuropathic pain (*BDNF, PRKCD*, *KCNQ2*, *CAMKIV, CREBBP*, *GFAP*, *IL15, GRIA2*, *GRM7*). We were able to verify direction of expression in seven of these genes (*BDNF, KCNQ2, GRM7*, *GRIA2*, *CAMKIV*, *IL15*, *GFAP*) using quantitative real-time polymerase chain reaction. Use of Western blot was only able to identify protein product for PRKCD and CAMKIV (Aim 3). Finally, in Chapter IV I provide a summary of the results, limitations, and future direction of this research.

Note: Italics are used for symbols referring to genes, no italics are used for symbols referring to proteins.

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CHAPTER II

PATHOPHYSIOLOGY OF CISPLATIN-INDUCED CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY

Neuropathic Pain

In the United States, there are more than 116 million people affected by chronic neuropathic pain, a public health issue that costs the economy over $600 billion dollars in healthcare expenses and lost work productivity each year (Institute of Medicine, 2011). Neuropathic pain is defined as pain from a lesion or disease of the somatosensory nervous system at any level (peripheral or central) and is recognized as chronic when it has persisted beyond 3 to 6 months (Jones, Lawson, & Backonja, 2016; Treede et al., 2008; Treede et al., 2015). Progressive research in chronic neuropathic pain has led to improved treatment options, but patients continue to report ineffective pain management and adverse effects on their activities of daily living and quality of life (Berger, Dukes, & Oster 2004; Cocito et al., 2006; Daousi, Benbow, Woodward, & MacFarlane, 2006; Werhagen, Hulting, & Molander, 2007; Wardach, Wagner, Jeong, & Holden, 2015). One patient population particularly affected by neuropathic pain is patients with chemotherapy-induced peripheral neuropathy (CIPN). CIPN is a common complication of chemotherapy treatment, and the pain and discomfort experienced by those with CIPN can limit the use of otherwise successful chemotherapeutic drugs.

Chemotherapy Induced Peripheral Neuropathy

Advances in early detection and treatment of cancer have led to almost 14.5 million cancer survivors in the United States (Seigel et al., 2012). As this number increases, so will the number of people who will suffer the side effects of the chemotherapeutic agents used to treat the disease. CIPN occurs in up to 68% of individuals receiving neurotoxic chemotherapy treatment (Argyriou, Bruna, Marmiroli, & Cavaletti, 2012; Cavaletti et al., 2013; Hershman et al., 2014; Kautio, Haanpaa, Kautiainen, Kalso, & Saarto, 2011; Mols, Beijers, Vreugdenhil, & van de Poll-Franse, 2014; Seretny et al., 2014). CIPN generally manifests with symptoms of pain, burning, numbness, and tingling in the extremities, but can additionally exhibit a number of impairments in the motor and autonomic nervous systems (Saif & Reardon, 2005; Smith et al., 2014). Muscle atrophy and weakness may result from motor neuron damage, and autonomic nerve injury can cause constipation, urinary retention, changes in blood pressure, and sexual dysfunction (Smith et al., 2014). Painful CIPN is reported in up to 40% of patients with CIPN, and can become chronic, persisting for months to years after chemotherapy (Kautio et al., 2011; Ventzel, Jenson, Jenson, Jenson, & Finnerup, 2016). Painful CIPN can be seriously debilitating, causing functional status impairment, decreased workplace productivity, and an overall decrease in quality of life (Mols et al., 2014; Travis et al., 2014). Traditional pharmacological treatments are ineffective for CIPN and continued symptomology can lead to dose reduction, delay, or even early termination of a potentially successful chemotherapy. The early withdrawal of chemotherapy due to the side effects of CIPN may increase patient mortality. Despite the known negative effects of painful CIPN on quality of life, few treatments and no preventative measures exist (Hershman et al., 2014).

The development of painful CIPN is contributed to alterations in spinal cord processing of pain, and modifications in the brain itself that can affect the way the body responds to painful (noxious) stimuli (Ji, Nackley, Huh, Terrando, & Maixner, 2018). Changes in the spinal cord are termed central sensitization and are well documented in the literature. The following discussion covers the processing of nociceptive information, pathophysiology and symptoms of cisplatin-induced CIPN.

Processing of Sensory Information

The terms nociception and nociceptor are derived from the Latin term "nocere," which means "painful." Nociception is the central and peripheral nervous systems processing of noxious stimuli (e.g. tissue injury) that activates nociceptors and their corresponding pathways, while pain refers to the subjective experience an individual perceives as the result of pathway activation (Dubin & Patapoutian, 2010). The experience of pain is subjective and highly complex, and the degree of pain that an individual will experience is not necessarily related to the amount of nociceptive input (Almeida, Roizenblatt, & Tufik, 2004; Bingel & Tracey, 2008). The perception of pain is the result of the processing of nociceptive information and is subject to significant modulations (pro-nociceptive and anti-nociceptive) that can be accomplished through spinal cord reflexes or supra-spinal cognitive factors (Bingel & Tracey, 2008). Descending pain modulation from the cortical areas to the brainstem and then the spinal cord contributes to the endogenous modulation of pain.

Properties of Sensory Nerve Fibers. Sensory information travels from the periphery to the central nervous system via peripheral sensory nerve fibers. The two major types of peripheral fibers are mechanoreceptors that preferentially detect non-noxious stimuli (touch and pressure) and nociceptors that detect noxious stimuli. Noxious stimuli are defined as those stimuli that have the potential to cause tissue damage. Examples of stimuli that inflict damage are intense mechanical pressure, extremes in temperature (<10°C and >40°C), and chemical substances, such as acids (Sneddon, 2018).

Mechanoreceptors. The different types of mechanoreceptors include the Aα and Aβ fibers. The Aα-mechanoreceptors are large (13 – 20 μM in diameter), myelinated fibers that conduct impulses at a speed of 80 to 120 m/s. The major function of Aα-fibers is in proprioception, interpreting information about movement from muscles and joints. Aβ- fibers are smaller than Aα, but at 6 to 12 μM in diameter, are still larger than nociceptors. The Aβ-mechanoreceptors are myelinated and conduct impulses at a rate of 35 to 90 m/s. As mechanoreceptors, the major function of the Aβ-fibers is to detect touch and pressure from the skin.

Nociceptors. Aδ-fibers and C-fibers are the two types of nociceptors. The myelinated Aδ-fibers are smaller than mechanoreceptors in diameter (1 – 5 μM) and conduct impulses at a speed of 5 to 40 m/s. Aδ-fibers are predominately mechanothermal fibers that can detect cold and heat on the skin. With a diameter of 0.02 to 1.5 μM, C-fibers are the smallest of all of the peripheral fibers. C-fibers are also the slowest conducting (0.5 – 2 m/s) peripheral fibers due to the fact that C-fibers are unmyelinated. There are various different types of C-fibers. These include C-fibers that are either polymodal (respond to mechanical, chemical, and thermal stimuli), respond solely to mechanical stimuli, or respond to mechanical, cold and heat (Sneddon, 2018). Another subset of C-fibers is termed "silent" because they only respond to heat or pressure after initial damage has led to the release of inflammatory molecules (Dubin & Patapoutian, 2010). Unlike the mechanoreceptors, nociceptors have free, or bare, nerve endings, branches of the primary neuron that are unmyelinated at nerve tips and serve as cutaneous nociceptors in the skin (Dubin & Patapoutian, 2010; Sneddon, 2018).

Ascending Pathways. The activation of the peripheral sensory nerve fiber propagates an action potential, which then travels the length of the of the peripheral nerve, through the nerve cell body (dorsal root ganglia [DRG]) to the second-order relay neuron located in the spinal cord dorsal horn. The peripheral nerve synapses with the second order neuron in the spinal cord. The axons of the second order neurons travel via the ascending sensory system to the brain stem, forebrain, and then to the cerebral cortex. The ascending sensory pathways are the major means by which the interaction of the body with the external environment is transmitted to the brain.

The ascending sensory pathways are classified according to anatomical location. The major ascending tracts are the spinothalamic, spinoreticular, spinomesencephalic, spinoparabracial, spinohypothalamic, spinocervical, and postsynaptic pathway of the spinal column (Almeida, et al., 2004). Each of these tracts carries the nociceptive impulse from the spinal cord to the brain, and the composition of the afferent fibers of each tract suggest the different aspects of pain with which each pathway is involved. The spinothalamic tract is involved in sensory-discriminative and motivational affective aspects or pain. The spinoreticular tract is involved in the motivational-affective characteristics of pain. The spinomesencephalic tract is capable of eliciting aversive behavior in the presence of noxious stimuli and some motor responses. The spinoparabracial tract contributes to the autonomic, motivational, affective, and neuroendocrine responses to pain. The spinohypothalamic tract contributes to motivational-affective, alert and neuroendocrine autonomic responses of visceral and somatic origin. The spinocervical pathway is involved in the motivational-affective, sensory-discriminative, and autonomic aspects of pain. The postsynaptic pathway of the spinal column is active in the motivational-affective and sensory-discriminative elements of pain (Almeida et al., 2004).

The spinohypothalamic tract is the ascending pathway that transmits the impulse to the lateral hypothalamus. The tract originates from the superficial dorsal horn, deep dorsal horn, and gray matter surrounding the central canal (Burstein et al., 1990; Burstein et al., 1991; Dado, Katter, & Geisler, 1994a,b). Nociceptive signals from second-order neuron in the spinal cord are projected to the lateral, prefornical, dorsomedial, suprachiasmatic, and supraoptic nuclei in the hypothalamus, where the second-order neuron synapses with the third order-neuron (Almeida et al., 2004).

Descending Pathways. Descending modulation of pain begins in the brain. Via a number of different synapses within areas of the forebrain and brainstem, the impulse from the stimulus returns to the spinal cord, where it is either potentiated (descending facilitation) or suppressed (descending inhibition; Millan, 2002). The lateral hypothalamus (LH) is part of the descending brain system that modulates pain in the spinal cord dorsal horn, although the full role of the LH in pain modulation is not known (Holden et al., 2014; Wardach, Wagner, Jeong, & Holden, 2016). In rats, both electrical (Dafny et al., 1996; Franco & Prado, 1996; Lopez & Cox, 1992) and chemical (Holden & Naleway, 2001; Holden, Van Poppel, & Thomas, 2002; Holden, Farah, & Jeong, 2005; Holden & Pizzi; 2008; Holden, Pizzi, & Jeong, 2009; Holden et al., 2014; Wagner, Banerjee, Jeong, & Holden, 2016; Wardach et al., 2016) stimulation produces antinociception in male (Behbehani, Park, & Clement, 1988; Dafny et al., 1996; Franco & Prado, 1996; Geraschenko, Horvath, & Xie, 2011; Holden et al., 2014; Safari, Haghparast, & Semnanian, 2009) and female rats (Holden & Naleway, 2001; Holden, Van Poppel, & Thomas, 2002; Holden, Naleway, & Jeong, 2005; Holden & Pizzi; 2008; Holden et al., 2014; Lopez & Cox,1992; Wardach et al., 2016) in a naïve (acute pain) state. Using the chronic constriction injury (CCI) model to induce neuropathic pain (Bennett & Xie, 1988), chemical stimulation of the LH produces antinociception in male (Holden et al., 2014; Wagner et al., 2016) and female rats (Holden et al., 2014; Wagner et al., 2016; Wardach et al., 2016). Clinically, deep brain stimulation of the hypothalamus has been used to treat cluster headaches (Akram et al., 2017;

Franzini, Ferroli, Leone, & Broggi, 2003).

The connection from the LH to the spinal cord dorsal horn can be either direct (Haghparast, Shafiei, Alizadeh, Ezzatpanah, & Haghparast, 2017, van den Pol, 1999, Wardach et al., 2016) or indirect via connection in the periaqueductal grey, nucleus raphe magnus, and A7 catecholamine cell group (Aimone & Gebhart, 1987; Aimone, Bauer, & Gebhart, 1988; Ezzatpanah, Babapour, Sadeghi, & Haghparast, 2015, Franco and Prado, 1996; Holden & Naleway, 2001; Holden, Van Poppel, & Thomas, 2002; Holden & Pizzi, 2008; Holden, Pizzi, & Jeong, 2009; Holden, Wagner & Reeves, 2018; Leite-Almeida, Valle-Fernandes, & Almeida 2006; Safari et al., 2009; Sim and Joseph, 1992; Wagner et al., 2016). The direct connection from the LH to the spinal cord dorsal horn is mediated via the orexin neuropeptides. Cell bodies of the orexin neuropeptides are located in the LH region of the hypothalamus (Peyron et al., 1998; van den Pol, Gao, Obrietan, Kilduff, & Belousov, 1998), blocking the orexin-1 receptor in the spinal cord dorsal horn of female rats results in antinociception (Wardach et al., 2016), providing evidence of a direct LH-spinal cord connection. The indirect connection is mediated through various pathways. In particular, LH stimulation releases the neuropeptide, substance P, that innervated spinally projecting noradrenergic neurons located in the A7 catecholamine cell group in the pons (Holden et al., 2002; Holden, Wagner, & Reeves, 2018). Another indirect connection involves a connection between the LH-induced antinociception via the nucleus raphe magnus resulting in the release of serotonin in the spinal cord dorsal horn (Holden et al., 2005).

Development of Painful Chemotherapy-induced Peripheral Neuropathy

Central Sensitization. The mechanism of central sensitization underlies the development of chronic neuropathic pain and can occur as the result of peripheral noxious stimuli, tissue injury, or nerve damage (Ji, Kohno, Moore, & Woolf, 2003). Central sensitization is described as an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability, synaptic efficacy, or a reduction of inhibition (Latremoliere & Woolf, 2009). Central sensitization works by recruiting previously sub-threshold impulses to nociceptive neurons, causing normally sub-threshold neurons to propagate action potentials that normally would not be generated. The recruitment of sub-threshold impulses induces changes in the size and overall threshold of the neuron receptive field (Latremoliere & Woolf, 2009). The effects of central sensitization can be short-term or longer lasting.

Central sensitization first came to attention when it was shown that post-injury pain hypersensitivity had a central nervous system component. Using un-anaesthetized rats that had been de-cerebrated to inhibit higher cerebral function, Woolf (1983) showed (1) mechanical pain threshold is stable after de-cerebration but lowered after a noxious insult; (2) exaggerated responses to a noxious thermal injury; and (3) an increase in spontaneous activity of the biceps femoris motor neuron efferents after thermal injury. These findings represent three cardinal signs of neuropathic pain, which are allodynia (pain from a normally non-painful stimulus), hyperalgesia (increased pain from a painful stimulus), and spontaneous pain (arising from spontaneous ectopic nerve activity).

Mechanism. There is no single defining molecular mechanism for central sensitization, rather, the development of central sensitization is contributed to several different factors (Latremoliere & Woolf, 2009). Peripheral inflammation and continued nociceptive input increases the release of neurotransmitters (glutamate, substance P, calcitonin gene-related protein [CGRP]) in the spinal cord. These neurotransmitters stimulate biological signaling pathways that produce a state of hyperexcitability in the spinal cord (Ji, Nackley, Huh, Terrando, & Maixner, 2018; Woolf 1983; Woolf & Salter 2000). Neuroinflammation is localized in the central nervous systems (peripheral and central) and is associated with various painful insults and pathologies, including trauma, surgery, and treatment with drugs such as anti-virals and chemotherapy (Ji, Xu, & Gao, 2014). During neuroinflammation, increased vascular permeability allows the infiltration of leukocytes and activation of glial cells (e.g. Schwann cells, astrocytes, and microglia) as well as the production of inflammatory mediators such as chemokines and cytokines that contribute to the development of central sensitization (Ji et al., 2018). For example, Toll-like receptor signaling plays an important role in immune response by recruiting molecules that regulate macrophage activation and has been shown to be involved in the development of painful CIPN after both paclitaxel and cisplatin treatment (Zhang et al., 2013; Park, Stokes, Corr, & Yaksh, 2014).

Activity-Dependent Central Sensitization. Activity-dependent central sensitization is pain hypersensitivity induced after intense noxious stimuli. There are two phases of activity-dependent central sensitization. The first is an early phosphorylation-dependent and transcription-independent phase resulting from rapid changes in the properties of glutamate receptors and ion channels (Latremoliere & Woolf, 2009; Woolf & Salter, 2000). The development and maintenance of central sensitization after nerve injury is dependent on the activation of the N-methyl-D-Aspartate (NMDA) receptor by glutamate, a primary neurotransmitter in the transduction of pain (Dubner & Ruda, 1992; Ji, et al., 2018; Ren, Hylden, Williams, Ruda, & Dubner, 1992). Under normal circumstances, the NMDA receptor is blocked by magnesium ions. The activation of primary nociceptors by sustained release of glutamate, substance P, or CGRP leads to membrane depolarization that removes the magnesium and activates the NMDA receptor. Activation of the receptor increases synaptic efficacy and triggers the influx of calcium into the cell. The calcium is now available to activate the intracellular signaling pathways that are responsible for the initiation and maintenance of central sensitization (Ji et al., 2003; Ji et al., 2018; Latremoliere & Woolf, 2009). For example, opening of calcium channels on the endoplasmic reticulum activate the PLC/PKC pathway involved in inflammation (Fagni, Chavis, Ango, & Bockaert, 2000; Yashpal, Fisher, Chabot, & Coderre, 2001). Glutamate binds to the NMDA receptor, creating an inward current that promotes the maintenance of central sensitization (Mayer, Westbrook, & Guthrie, 1984). As this inward current is being created, a shift in glutamate receptor subtypes mGluR2 and mGluR3 into mGluR1 takes place, increasing the amount of calcium in the cell. Intracellular pathways activated by the phosphorylation of the glutamate receptors help to maintain central sensitization.

The later, longer lasting phase of central sensitization is transcription dependent. Transcription refers to the beginning of gene expression which is copying, or transcribing RNA from DNA. This phase is responsible for driving the synthesis of new proteins that contribute to the longer lasting form of central sensitization that can be seen in many pathological conditions, including neuropathic pain (Latremoliere and Woolf, 2009; Woolf, 2011). For example, during the development of neuropathic pain, DRGs in both injured and non-injured neurons experience changes in transcription that alter membrane properties, growth, and transmitter function (Costigan et al., 2002; Latremoliere & Woolf, 2009; Obata et al., 2003; Obata et al., 2004; Romanelli & Esposito, 2004; Xiao et al., 2002).

Windup. Windup is a frequency-dependent increase in spinal cord neuron excitability induced by electrical stimulation of C-fiber afferents that shares properties with central sensitization and can lead to central sensitization, but wind-up and central sensitization are distinct (Herrero, Laird, & Lopez-Garcia, 2000; Woolf & Thompson, 1991). Windup is an example of the increase in excitability that occurs prior to the manifestation of central sensitization but disappears when the membrane potential return to its normal resting state, within ten seconds of stimulus cessation (Latremoliere & Woolf, 2009).

Windup occurs in the spinal cord when the action potential discharge elicited by a sequence of identical strength, low-frequency C-fiber stimuli increases with each additional stimulus (Mendell & Wall, 1965). The continued C-fiber bombardment of the spinal cord stimulates kinase production which in turn increases both glutamate and NMDA receptors, creating an additive membrane depolarization (Eide, 2000; Herrero, Laird, & Lopez-Garcia, 2000). Chronic C-fiber bombardment creates anatomical changes in the spinal cord dorsal horn, termed “sprouting,” by disorganizing the processing of impulses (Woolf, Shortland, & Coggeshall, 1992). During sprouting the laminae of the dorsal horn become disordered and the boundaries of the different laminae begin to structurally reorganize, disrupting normal pain processing (Woolf, Shortland, & Coggeshall, 1992).

After peripheral nerve injury, degeneration of C-fiber terminals in spinal cord lamina II, gives A-β fibers the opportunity to “sprout” into laminae II towards the C-fibers, generating A-β fiber mediated pain (Farajidavar, Towhidkhah, Mirhashemi, Gharibzadeh, & Behbehani, 2006; Latremoliere & Woolf, 2009; Lekan, Carlton, & Coggeshall, 1996; Woolf, Shortland, & Coggeshall, 1992). A-β fiber mediated pain can be explained through structural reorganization, increased excitability, and decreased inhibition (Woolf & Doubell, 1994). Structural reorganization refers to myelinated A-β fibers sprouting from their normal terminal areas in spinal cord laminae III and IV into lamina II to make contact with nociceptive neurons (Woolf, Shortland, & Coggeshall, 1992; Woolf & Doubell, 1994). Increased excitability occurs because previously sub-threshold impulses are now able to generate action potentials. Decreased inhibition is contributed to apoptosis of inhibitory interneurons resulting from a NMDA receptor induced excitotoxicity that develops over time (Latremoliere & Woolf, 2009; Scholz et al., 2005).

Cisplatin

Cisdiamine dichloroplatinum (cisplatin) is a platinum-based chemotherapeutic agent used as a first-line treatment for cancers of breast, lung, ovary, testis, bladder, head/neck, and endometrium (Allen, 1991; Lee et al., 2014; Manji, 2011; McKeage, 1995; Seaver et al., 1994*)*. The drug treats cancer by damaging DNA of the dorsal root ganglia (DRG) and forming both intra- and interstrand crosslinks that block replication in tumor cells (Alaedini, Xiang, Kim, Sung, & Latov, 2008; Quasthoff & Hartung, 2002). The blocked replication sets off DNA repair mechanisms that activate apoptosis of the cell (Aldehini et al., 2008; Fisher, McDonald, Gross, & Windebank, 2001; Gill & Windebank, 1998; McDonald, Randon, Knight & Windebank, 2004; Quasthoff & Hartung, 2002). Cisplatin is neurotoxic at high doses.

Cisplatin-induced Peripheral Neuropathy

The first reports of cisplatin-induced CIPN occurred in the 1970s, shortly after the drug was first introduced (VonHoff et al., 1979; Walsh, Clark, Parhad, & Green, 1982). Some degree of peripheral neuropathy is reported in more than half of patients that receive a total cumulative dose of cisplatin ranging from 225 – 500 mg/m^2 (Argyriou, et al., 2012; Argyriou, Kyritis, Makatsoris, & Kalofonos, 2014). Known risk factors of cisplatin-induced CIPN include prior or simultaneous administration of taxanes, single and cumulative cisplatin dose levels, and pre-existing peripheral neuropathy (Argyriou et al., 2014).

Evaluation of cisplatin-induced CIPN is heavily based on clinical assessment. Signs and symptoms include: Diminished perception of vibrations, loss of tendon reflexes, and an uncomfortable burning or prickling sensation (paresthesia), beginning in the lower extremities

(Quasthoff & Hartung, 2002; Thompson, Davis, Kornfeld, Hilgers, & Standefer, 1984). The paresthesia develops in a symmetrical 'stocking-and-glove' distribution pattern that starts in the distal ends of the longest axons in the body.

Mechanism. The mechanism of toxicity that leads to peripheral neuropathy is most likely due to platinum-DNA binding in the DRG (McDonald et al., 2004), but is also based on the disturbance of cellular metabolism and axo-plasmatic transport (Quasthoff & Hartung, 2002). Cisplatin accumulates in the DRG via fenestrated capillaries that allow passage of molecules between circulation and the extracellular fluid of the DRG (Gill & Windebank, 1998). Levels of platinum in the body have been found to be much higher in the DRG and other peripheral nerve structures than in the brain and spinal cord, suggesting that platinum does not cross the blood brain barrier (Fischer et al., 2001; Seaver et al., 1994) leaving the motor neurons and other central nervous system neurons without direct exposure to toxic levels of cisplatin. It has been suggested that this could be part of the reason why patients with cisplatin-induced CIPN rarely develop motor neuropathy (Thompson, et al., 1984; Seaver et al, 1994).

Current CIPN treatment strategies are focused on the relief of symptoms, the most common being pain. Therefore, many treatment efforts are focused on the development and maintenance of central sensitization but are met with limited success. The development of CIPN can be contributed to a myriad of insults that lead to the development of central sensitization (Ma, Kavelaars, Dougherty, & Heijnen, 2018). For instance, cisplatin binds to nuclear DNA in the DRG causing DNA damage and leading to apoptosis through cell cycle changes partially brought about through upregulation in tumor suppressor protein, p53 (Fisher et al., 2001). It has also been shown that cisplatin induces mitochondrial dysfunction in peripheral sensory fibers by binding to mitochondrial DNA and inhibiting the production of mitochondrial proteins, thus leading to impaired energy production (Podratz et al., 2011). Due to the energy deficits in the sensory fibers, the neurons are no longer able to sustain normal cellular activity and activities such as sodium-potassium pump dysfunction that lead to the development of central sensitization (Ma et al., 2018).

Another insult associated with the development of central sensitization is oxidative stress. Oxidative stress is described as the imbalance between free-radical production and ability of the body to produce antioxidants to detoxify their effects. The mitochondria are an important source of endogenous free-radicals and disruption of normal mitochondria function leads to dysregulation of free-radical production and generation of oxidative stress (Brand et al., 2004). The high amounts of phospholipids and mitochondria along with weak antioxidant defenses leaves the peripheral nervous system susceptible to chemotherapy-induced oxidative damage (Ma et al., 2018). The sustained nervous system damage that results from the effects of cisplatin in the DRG lead to the development of chronic pain via central sensitization.

The Lateral Hypothalamus in Painful Cisplatin-induced Peripheral Neuropathy

Little is known about how LH might facilitate the development of painful CIPN. As the location of a third-order neuron in the spinohypothalamic ascending pathway, the LH is a unique and valuable area for study. As described previously, the induction of cisplatin-induced CIPN is the result of cisplatin accumulation in both nuclear and mitochondrial DNA in the DRG, the anatomical location of the nociceptor cell body. The central sensitization resulting from cisplatin accumulation results in a higher than normal amount of nociceptive and previously non-nociceptive impulses being transmitted. In the dorsal horn of the spinal cord, the peripheral nociceptor synapses with the second order neuron. The axon of the second order neuron decussates to the contralateral side of the spinal cord and then transmits the ascending sensory information via the spinohypothalamic tract to the LH. It is not known if central sensitization-like changes happen in the LH, but there is evidence of neuronal modification after painful injury, making the idea of tertiary changes in the LH after cisplatin-induced CIPN a possibility. For example, there is evidence of an upregulation in glutamate receptors in the cortex within days of sciatic nerve injury in mice (Kim et al., 2016).

Using the RNA-sequencing method to measure gene expression in the prefrontal cortex of mice receiving the spared nerve injury model of pain, several genes with known roles in chronic pain were found to be differentially expressed between nerve-injury and sham control animals (Alvarado et al., 2013). The gene expression differences in the NMDA glutamate receptor, glial fibrillary acidic protein, and voltage-gated sodium channel, type 1 were present six months after injury. The findings of this study are suggestive that gene expression changes in the brain are maintained following peripheral nerve injury.

Other gene expression studies using a mouse model of paclitaxel-induced CIPN show changes in the brain that could possibly be involved in neuropathic pain development. Using real-time quantitative polymerase chain reaction (qPCR) of the anterior cingulate cortex of mice, researchers discovered an increase in the expression of GABA transporter-1, a gene that transports GABA to and from the synaptic cleft (Masocha, 2015a). Significant increase in gene expression were also detected in the glial fibrillary acidic protein gene an astrocyte marker, as well as significant increases in certain glutamate receptor subunits (Masocha, 2015b).

Altered gene expression in the LH has been reported using a genome-wide microarray approach to identify LH responses to chronic activation of the mu opioid receptor with escalating doses of morphine in mice (Befort et al., 2008). The focus of this study was LH changes associated with addictive behaviors concerning drug abuse, but it should be noted that the researchers did find altered neurotransmission in LH after chronic intraperitoneal injections of morphine, providing evidence that the LH is an area able to undertake changes in plasticity.

In this dissertation, the overarching goal is to add to the scientific knowledge regarding the relationship between gene expression in the LH and cisplatin administration and how this relationship contributes to the development of allodynia, a sign of painful CIPN. The aim of this study is to use a pre-clinical study to determine key genes that may provide at least part of the answer as to why some individuals develop allodynia post cisplatin treatment and others do not. To our knowledge, this is the first study to investigate the relationship between the LH and painful CIPN development.

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CHAPTER III

A CASE STUDY OF USING GENOMIC TECHNOLOGY IN NURSING RESEARCH

Abstract

The current shift in healthcare towards a model of precision health exposes nurses to a variety of genomic tests and technology while offering the opportunity to integrate genomics into nursing research. The objectives of both precision health and nursing science are similar in that both are inclusive of an individualized, patient-centered approach to care. Yet, standard nursing education provides limited training on genomic methodologies and approaches to the analysis and interpretation of genomic data. Our rationale for this manuscript is to provide one example of how genomic technology is integrated into nursing research. Our purpose was to describe a case example of research utilizing genomic technologies to examine gene expression in the lateral hypothalamus (LH) of mice before and after administration of cisplatin. We describe our experience of measuring and validating gene expression in the LH before and after cisplatin treatment. The genomic technology that we describe include microarray analysis, validating our results using quantitative real-time polymerase chain reaction (qPCR), and Western blot. We include a brief overview for each technique, along with advantages and disadvantages of each procedure. As a result of understanding the rationale and science behind the methods used in genomic research, nurses can interpret and apply genomic results in the clinical setting and provide the highest standard of precision healthcare to patients.

Introduction

In this era of precision health, nurses are increasingly exposed to a vast array of genetic tests and technologies in clinical and research settings. Precision health considers individual variability in areas such as genes, environment, and lifestyle when determining the best preventative or treatment measures (Collins & Varmus, 2015). The concept underlying precision health is closely related to the scope and practice of nursing, which includes an individualized, patient-centered approach to care. An area of precision health with great potential for growth in nursing research is the integration of genomics (Lee, Gill, Barr, Yun, & Kim, 2017; Lemoine, 2014; Wickersham & Dorsey, 2017). The Genomic Nursing State of the Science Advisory Panel has developed a blueprint for nursing research in genomic science with the goal of identifying how nurses can use genomic science to address research gaps (Calzone et al., 2013). The blueprint outlines genomic research topic areas that fall into the specific categories of the National Institute of Nursing Research strategic plan. For example, a nurse researcher can use genomic methods to investigate changes in biochemical pathways involved in symptom development or management that can be exploited to improve patient quality of life (Calzone et al., 2013; Cashion et al., 2013; Dorsey et al., 2009; Founds, Conley, Lyons-Weiler, Jeyabalan, Hogge, & Conrad, 2009).

As a profession, nurses are well-positioned to bring genetics and genomics into research and clinical practice (Calzone et al., 2010; Ferranti, Grossman, Starkweather, & Heitkemper, 2017). To do this, nurses must understand the technologies being used to conduct research as well as interpret and apply research findings (Lee, et al., 2017); however, standard nursing education provides limited instruction regarding genetics/genomics methodologies and approaches to analysis of genomic data (Anderson, Alt-White, Schaa, Boyd, & Kasper, 2015;

Camak, 2016; Cashion, Driscoll, & Sabek, 2004). The purpose of this manuscript is to explain common approaches used in genomic research from the viewpoint of the author's doctoral dissertation research examining gene expression in the lateral hypothalamus (LH) of mice before and after administration of the chemotherapy drug, cisplatin. Methods of evaluating gene expression (microarray) and validation of microarray results using quantitative real-time polymerase chain reaction (qPCR) and Western blot are discussed.

Background and Significance

The integration of genomics research in nursing, has become the integration of omics methodologies into nursing research. The term "omics" refers to the incorporation of certain aspects of research areas that include genomics, proteomics, epigenomics, metabolomics, examination of the microbiome, and transcriptomics. Through the use of omics research, nurses are able to merge different aspects of biological knowledge to represent the complexity and diversity of living systems throughout the lifespan (Pierce & Henly, 2017).

The continued integration of omic science into nursing requires a sound understanding of the molecular foundations underlying the science. To understand the anatomy and physiology of symptoms and disease, nurses must also understand the genes and proteins that give rise to these attributes and be exposed to the background information necessary to understand the techniques. The basis for omics research is called the central dogma of molecular biology (Crick, 1970) and is a framework for understanding how sequence information is exchanged within the cell. There are two important activities in the central dogma: transcription and translation. Transcription takes place in the nucleus of the cell and is defined as the synthesis of RNA from a DNA sequence. Translation is the production of a protein from the transcribed RNA sequence, termed messenger RNA (mRNA) or transcript, and occurs in the cytoplasm (Figure 3-1).

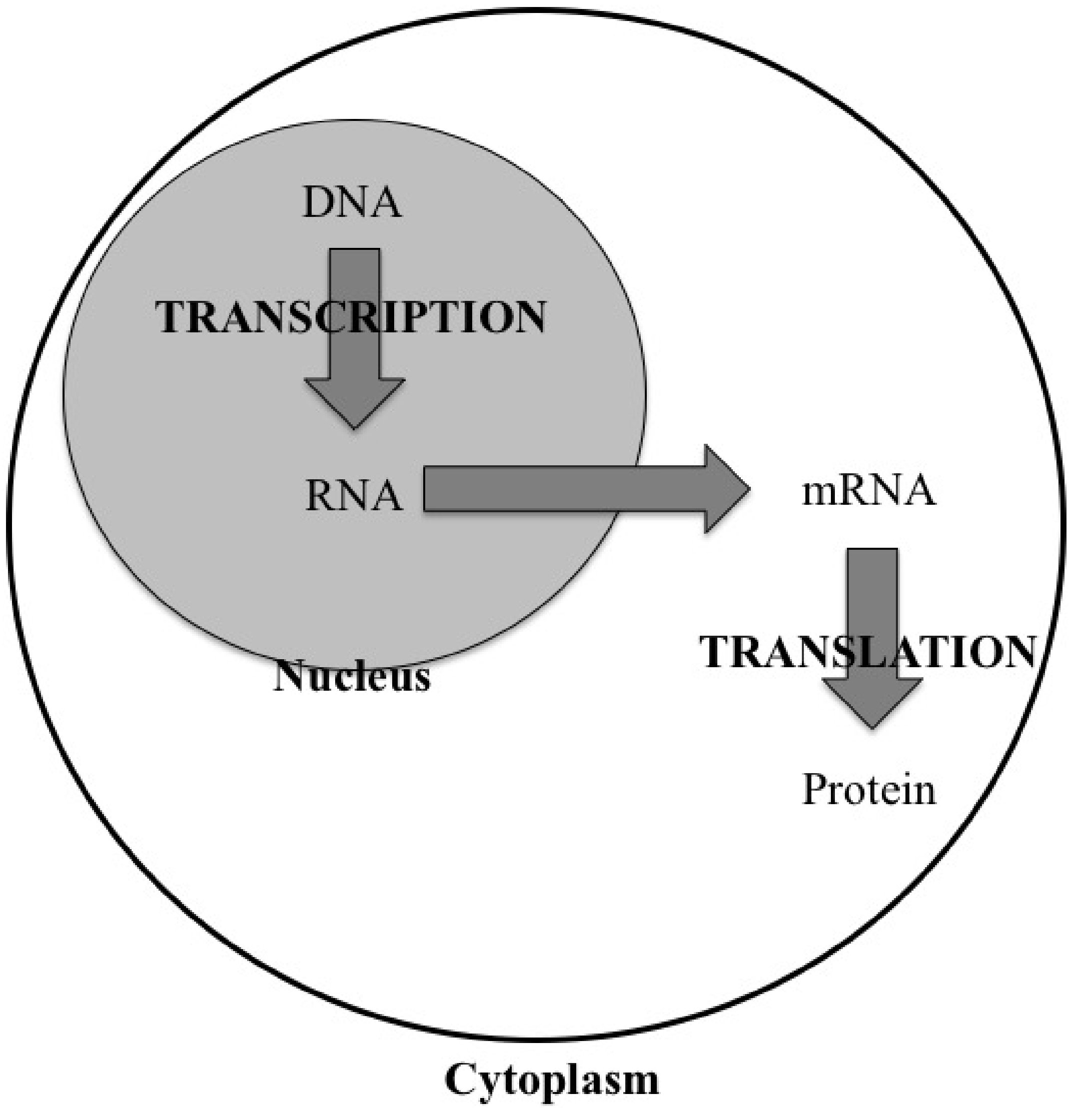

Figure 3-1. The central dogma of molecular biology.
The central dogma of molecular biology is a framework for explaining how genetic information is exchanged within the cell. The DNA sequence is transcribed into total RNA. Prior to leaving the nucleus, intron sequences are removed from the RNA and only the exon sequences enter the cytoplasm as mRNA. Once in the cytoplasm, the mRNA is translated into protein.

When measuring sequence information, the experimental approach is guided by the research question. In this paper, we will use the author's dissertation study to illustrate use of one omics method and validation of study findings as a means of answering questions about the manifestation of disease and/or side-effects of treatment. The purpose of the dissertation research was to 1) identify LH-derived gene expression differences between two strains of mice, and 2) examine how these differences might increase knowledge regarding differential allodynia (feeling pain from a normally non-painful stimulus; a side-effect of painful chemotherapy-induced peripheral neuropathy) development after cisplatin treatment. The omics technology used for the exemplar was transcriptomics (Ferranti et al., 2017; Wang et al., 2009).

A transcript refers to the sequence of mRNA that has been copied, base for base, from the original DNA sequence (Draghici, 2012). Transcripts are part of the flow of information that will eventually be translated into a protein. The transcriptome refers to the set of all the RNA transcripts (genes) found in a particular cell or tissue type at a certain point in time. Because gene expression is tightly regulated, genes are only expressed at times when a distinct function is useful. For instance, in times of stress, your transcriptome will contain a greater amount of expressed mRNA transcript from the gene that codes for norepinephrine. Transcriptome data can be used to identify alterations in a set of candidate genes or identified (canonical) pathways at the level of mRNA and can be helpful in predicting susceptibility to disease or providing insight on novel targets for therapeutic intervention (Harrington, Rosenow, & Retief, 2000; Kurella et al., 2001; Noordewier & Warren, 2001; Wickersham & Dorsey, 2017).

To illustrate the use of omics in nursing research, we provide an exemplar describing the examination of the LH transcriptome in mice before and after cisplatin treatment in response to the difference in allodynia development between the strains of mice. The purpose was to compare the results of behavioral testing for allodynia development with LH gene expression in C57BL/6J and A/J mice. The overall goal was to understand the contribution of gene expression changes in the LH to behavioral differences exhibited by C57BL/6J mice developing allodynia at a faster rate than A/J mice. A common side effect of cisplatin treatment is chemotherapy-induced peripheral neuropathy (CIPN). In preclinical research, mouse models are commonly used due to their small size and the ease with which mice can be bred. Over 95% of the mouse genome is similar to that of humans making genomic research done with mice applicable to human disease (Hardouin & Nagy, 2000). Allodynia, a common sign of neuropathic pain, is measured in mice as a surrogate for CIPN. We used microarray analysis to examine changes in gene expression in response to the development of allodynia post cisplatin. We then validated select genes using qPCR to confirm gene expression (direction and magnitude). Lastly, we performed Western blots to detect the presence of protein in LH tissue as confirmation of whether differences in gene expression between the strains of mice translate into differences in protein.

Microarray

The use of microarray technology allows for a non-biased approach in identifying differentially expressed genes in a particular tissue or cell type in response to a physiological challenge by simultaneously measuring gene expression in up to tens of thousands of genes (Hindmarch, 2013). Nurse scientists have used microarray technology in discovering that the gene “giant axonal neuropathy” is differentially regulated in mice that receive the nucleoside reverse transcriptase inhibitor (NRTI), stavudine, identifying this gene as a potential target for treatment of NRTI-induced neuropathy (Dorsey et al., 2009). Microarray has also been used by nurse researchers to examine changes in placental tissue gene expression in women during the first trimester of pregnancy to identify possible biomarkers for the development of preeclampsia (Founds et al., 2009).

Array Design. We used the GeneChip® Mouse Genome 430 2.0 array from Affymetrix (Carlsbad, CA, USA), that simultaneously measures expression for over 34,000 mouse genes (Figure 3-2). The Affymetrix GeneChip® (referred to as array or microarray from this point forward) is made of laboratory generated RNA sequences (oligonucleotides or probes) attached to a piece of quartz and enclosed in a plastic case (Dalma-Weiszhausz, Warrington, Tanimoto, & Miyada, 2006; Hindmarch, 2013). There are close to a million probes on the array, and the average length of each probe is 25 nucleotides (the unit molecules of RNA). In our experiment, we used one array for each mouse.

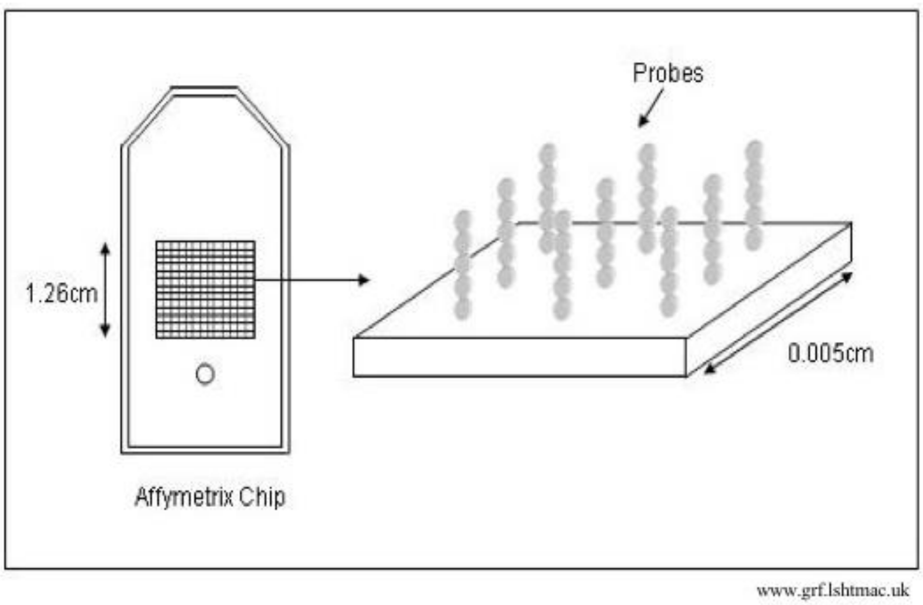

Figure 3-2. The Affymetrix GeneChip®
Representation of the Affymetrix GeneChip® showing the shape and design of the quartz wafer inside of the plastic case that encloses it as well as the organization of the probes, or single stranded DNA sequences.

Experimental Workflow. Our microarray experiment began with isolation of total RNA from previously dissected LH tissue samples (Figure 3-3). Next, we performed a massive replication of the RNA, making multiple copies of each gene, ensuring enough RNA to bind the probes on the array. The nucleotides were then labeled with biotin (a small protein that combines with detection molecules) and fragmented, in preparation for hybridization of the RNA to its complimentary probe sequence on the chip.

The labeled and fragmented RNA samples were injected into the arrays for the 16-hour hybridization process. After hybridization, the arrays were washed with buffer to remove the unbound nucleic acids. The biotin-labeled RNA bound to the arrays was then stained with a fluorescent detection molecule (Harrington et al., 2000). The microarray chip was scanned via a laser passed over its surface to excite the fluorophores bound to the hybridized nucleic acids. The resultant fluorescent values were converted into pixel values that were then translated into numerical values to represent fluorescent intensities (intensity values; Dalma-Weiszhausz et al., 2006). The intensity values were then normalized. Normalization includes correction for differences in background signals from the scan, effectiveness of hybridization, and non-specific binding across all arrays used in the experiment. Normalization is based on the assumptions that all microarray chips within the same condition are similar and that majority of the probes on the chip will not experience expression changes (Draghici, 2012).

Microarray statistical analysis was conducted using R software packages developed by Bioconductor (www.bioconductor.org). The normalized microarray data were analyzed by both one-way and two-way ANOVA using the Limma R package (v.1.48.0; Smyth 2004) to determine the effects of both treatment and strain on the development of allodynia in mice. To correct for error inflation due to multiple statistical testing, the raw p-value was adjusted into the

False Discovery Rate (FDR) using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). We used an FDR value at the level of ≤ 0.10 and a gene expression fold change of ± 1.5 as cutoff points for significance.

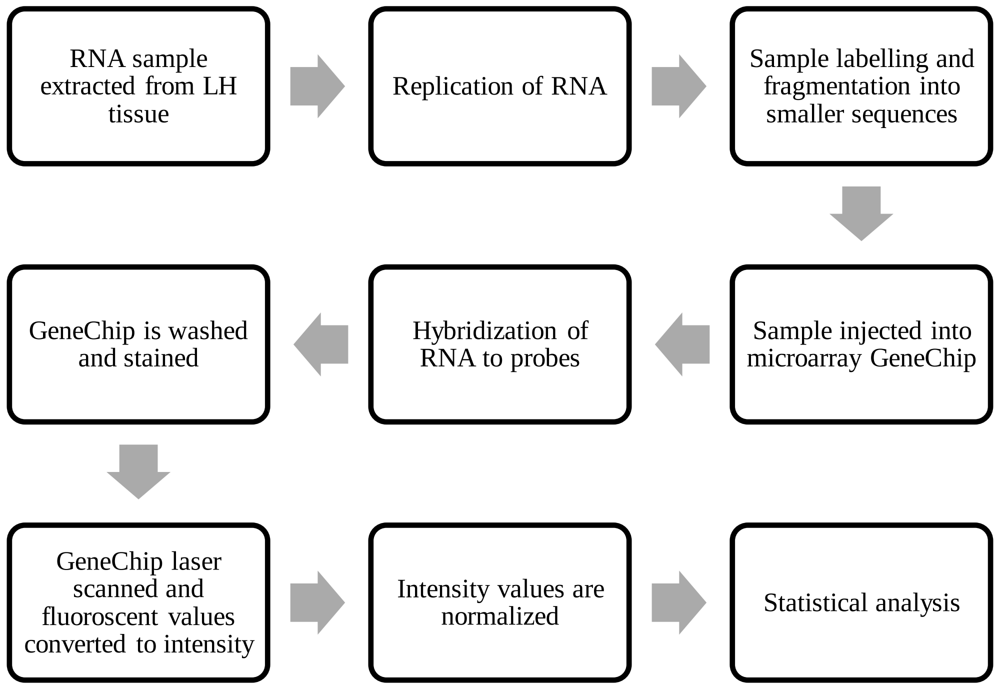

Figure 3-3. Flowchart of microarray experiment.
After extracting total RNA from LH tissue, we replicated the RNA to create multiple copies of each gene, and then we labeled the sample with biotin to prepare for the next steps. The sample was then injected into the GeneChip and allowed to hybridize to the probes overnight in the hybridization oven. The GeneChip was then washed with buffer and stained with a detection molecule. Next, the chip was scanned with a laser to excite the detection molecule and the resultant fluorescent values were converted into intensity values. We then used to the normalized intensity values to perform the statistical analysis of the differences between the treatments and strains.

Further Characterization of Genes. To identify canonical pathways and functions altered in our experiment we analyzed the list of differentially expressed genes from the microarray using Ingenuity Pathway Analysis (IPA, Ingenuity Systems). We wanted to know which biochemical signaling pathways, if any, were closely associated with differentially expressed genes in our dataset. IPA core analysis interprets the data in context of biological pathways, processes, and molecular networks (http://www.ingenuity.com). The top canonical pathways contain genes related to a particular biological process and are created based on the genes identified in our microarray analysis results. The upstream analysis is a list of what gene regulators (genes involved in the expression of one or more other genes) in the data set contribute to the expression of other genes in the dataset. Top diseases and biological functions are determined by combining the canonical pathways and upstream analysis to make predictions about biological functions into a composite view of the physiological effects of gene expression changes. This stage of the characterization is where potential mechanisms behind a phenotype, potential drug targets, or biological impact of upstream molecules are identified. Using previously published associations between the genes identified from the microarray analysis, IPA can also compile networks of interconnectivity that are independent of the canonical pathways. Finally, IPA creates a list of the top up and down-regulated genes found in the analysis. In our study, we used pathway analysis to identify the top differentially enriched canonical biological signaling pathways between C57BL/6J and A/J mice (Figure 3-4) as well as identify select genes for further validation.

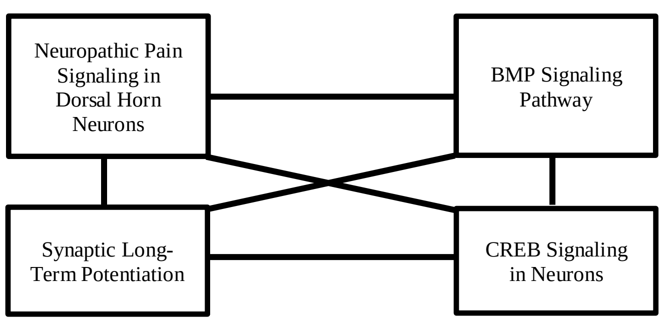

Figure 3-4. Four of the top canonical pathways.
The lines show the relationship between the genes in the pathways. Each pathway is related to all of the others, meaning that at least one of the differentially expressed genes from our dataset is involved in each of these pathways. This relationship helps explain why a change in one gene can lead to more than one symptom.

Advantages, Disadvantages and Considerations of Microarray Technology. There are both advantages and disadvantages as well as features to consider when using microarray technology. The use of microarray is hypothesis-generating rather than hypothesis-driven (Kurellea et al., 2001) offering a non-biased view of the entire transcriptome of the LH tissue. Investigating the entire transcriptome without a hypothesis allows for novel discoveries. Through our microarray experiment, we have increased understanding of gene expression changes in the LH of A/J and C57BL/6J mice both before and after four weeks of cisplatin treatment.

By performing a microarray analysis, we were able to examine all of the RNA transcripts being expressed at the time of tissue harvest, which in the case of our study was either before (represented by mice who were naïve to treatment) or after four weeks of cisplatin treatment. An advantage of the ability to perform this non-biased approach is that we could appreciate every gene that was being expressed at those particular points in time, but the disadvantage was that we could only see changes that were present at the time of harvest. Therefore, we were not able to identify any gene expression changes within the first three weeks of cisplatin treatment that may be important in the development of allodynia.

Another consideration concerns the cutoff criteria used to determine significance of the gene expression changes. The FDR set for our experiment was ≤ 0.10. With the high number of comparisons being made from the experiment, there was a high possibility of a false positive because there were more statistically positive genes than expected by chance, which can lead to rejection of the true null hypothesis. To account for error of inflation due to multiple statistical testing that is experienced from the comparisons on the microarray, the FDR was adjusted using the Benjamini-Hochberg method (1995).

When using microarray, results are limited to the gene probes pre-embedded on the array. Therefore, our analysis was restricted to known genes and did not allow for the discovery of new genes. In addition, the resolution of the microarray is 8-bit with only 256 levels of gray used to store one pixel (Draghici, 2012). Therefore, some of the genes that were highly up-regulated (high intensity values) or highly down-regulated (low intensity values) might not have been captured as they were beyond the capabilities of the microarray. Due to these intrinsic issues within the design of the microarray, we validated our results using a second method of gene expression analysis.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Nurse researchers can use qPCR to verify the results of microarray analyses. qPCR differs from microarray in that qPCR uses a candidate gene approach, quantifying the gene expression level of chosen target genes as opposed to every gene in the sample (Bachman, 2013; Rio 2014). Nurses have used qPCR to study gene expression associated with inflammatory pain, NRTI-induced neuropathy, first trimester preeclampsia, and pain associated with spinal cord injury (Dorsey et al., 2009; Founds, Terhorst, Conrad, Hogge, Jeyabalan, & Conley, 2011; Wang, Hanza, Gordon, Wahl, & Dionne, 2007; Wu, Renn, Faden & Dorsey, 2013). Through an understanding of the process of qPCR, nurses can better appreciate and apply the results of genomic studies into clinical practice.

A standard PCR is a biochemical reaction that uses controlled cycles of heating and cooling in the presence of DNA synthesizing enzymes to amplify a target sequence of DNA (Donaldson, 2013). This type of reaction is typically used for genotyping, sequencing, forensic investigations, and paternity testing (Cashion, et al., 2004; Donaldson, 2013). Results of a standard PCR are obtained after the reaction has been completed. The qPCR reaction differs from standard PCR in that qPCR allows for precise quantification of DNA, even if the starting concentration of the sample is a very small amount. The quantification is accomplished through monitoring the amplification of a target sequence, termed amplicon, in real-time using fluorescent technology. Using real-time qPCR, the user can visualize the amplicon as the reaction progresses (Cashion, et al., 2004). How quickly the amplicon reaches the threshold detection cycle correlates with the amount of starting material present in the original sample. Reaching threshold at an earlier cycle means that there is more starting material in the sample than there would be at a later threshold cycle. A common use for qPCR is measuring gene expression. In our study we used qPCR to validate the expression of certain genes chosen based on a documented link between the gene and the development of pain.

Experimental Workflow. Similar to microarray, the first step of our qPCR reaction was the isolation of total RNA from LH tissue (Figure 3-5). The single-stranded total RNA was next converted to more stable double-stranded complementary DNA (cDNA) using the process of reverse transcription (Taylor et al., 2010). The reason for performing this step is that the qPCR reaction requires DNA. We performed the reverse transcription of RNA into cDNA by first combining total RNA with different random primer sequences and nucleotides necessary to build the new cDNA strand. Then, using controlled temperature cycles, the secondary structure of total RNA was unwound, and cDNA reverse transcribed (Bachman, 2013).

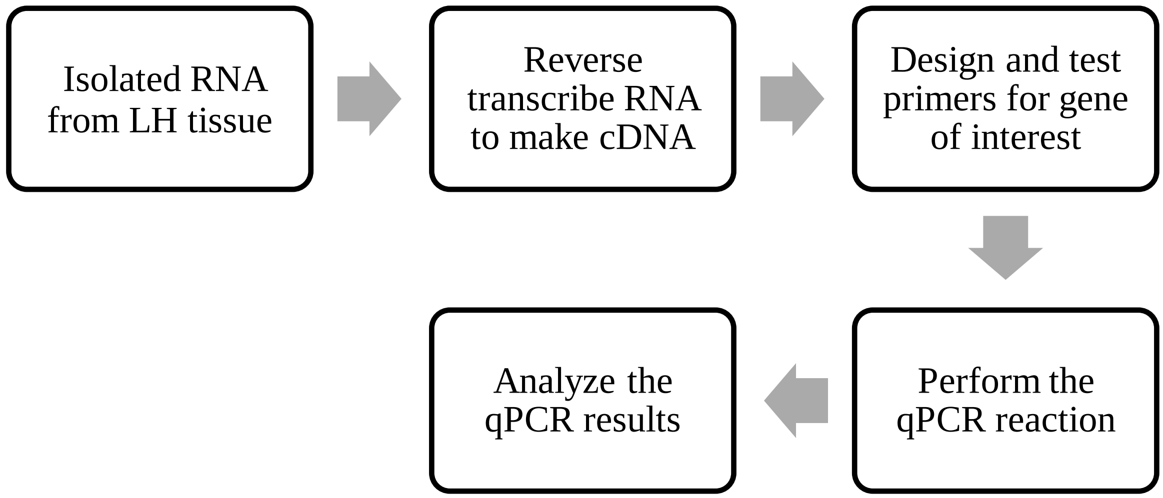

Figure 3-5. Flowchart of real-time qPCR reaction.
After isolating RNA from LH tissue, the single stranded RNA was reverse transcribed into more stable, double stranded complementary DNA (cDNA). In preparation of the qPCR reaction we next specifically designed and tested primers for each of our genes of interest. After we had ensured that each primer would amplify only the gene of interest, we were ready to perform the qPCR experiment. Once the experiment was complete, we analyzed the results to determine the amount of each gene of interest in the LH tissue.

Primer Design. Primers are short (15 – 30 nucleotides) single-stranded sequences of DNA designed to complement a specific sequence within the gene of interest. The primer sequences must be designed specifically because a non-specific primer can lead to false annealing (combination of the primer with its corresponding DNA sequence), thus, amplification of sequences other than that of interest. The success of the qPCR reaction is dependent on primer design. Certain specifications, such as amplicon length, the nucleotide composition, and the temperature at which the primer attaches to the amplicon help to increase the specificity of the primer (Rio, 2014; Taylor et al., 2010). Each gene of interest requires a forward and a reverse primer, each primer being complementary to one strand of the double stranded target DNA sequence. The primers bind onto the target DNA, (one primer to each strand, at opposite ends of the sequence to be amplified) and act as starting points for the DNA polymerase enzyme to build a new strand. Once designed, all primers should be compared against a sequence database to ensure that only the gene of interest is amplified. We used the BLAST algorithm, a program designed by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/).

Primer Testing. Prior to performing our experimental qPCR, we assessed the reaction conditions to ensure optimization of each target gene by evaluating for efficiency and specificity of the qPCR reaction. In order for a reaction to be efficient, there should be an exact doubling of the amount of PCR product produced during the linear phase of the reaction. To evaluate the efficiency of our reaction, we performed a 2- fold water dilution series, creating eight separate cDNA concentrations to verify that the amount of product doubled with each cycle of PCR. Ideally, the amplification curves produced by the dilution series will be evenly spaced on the data report with maximum efficiency of the reaction (Taylor, 2010). If the efficiency of the reaction is poor, it means that the cDNA is of a poor quality and that the product is not being produced at an optimal rate.

We established the specificity of the reaction by analyzing the melting curve created during the qPCR reaction with its corresponding primer. The melting curve is produced at the end of all PCR amplification cycles and establishes how well the primer anneals to the target sequence. If the primer is optimal for the reaction, the melting curve is displayed a single sharp peak (Figure 3-6). If the primer targets more than one sequence, the melting curve will have more than one peak, and that primer is redesigned. Primer design and testing can be the most time-consuming steps of qPCR.

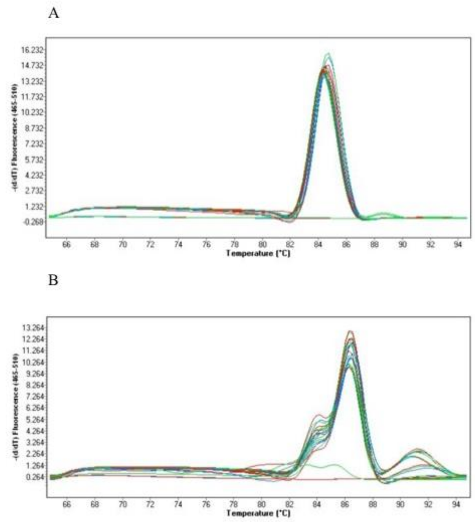

Figure 3-6. Melt curves to evaluate primer specificity.
Only one peak was present in the top test (A), meaning this primer was optimal for the reaction. More than one peak was present in the bottom test (B), meaning the primer had more than one target and the results of amplification may not be specific to the target of interest. This primer was not used for the reaction.

qPCR Reaction. After determining the efficiency and specificity of our primers, we were ready to perform the experimental qPCR reaction. We began by combining a small amount of cDNA with the forward and reverse primer from one target gene, and the fluorescent technology used to detect the target sequence into the individual wells of a 96-well plate. The plate was then covered, analyzed, and the relative abundance of each sequence was computed using Roche LightCycler® 480 Relative Quantification software version 1.5.0 (Roche Life Science). The amount of fluorescence was quantified after each cycle of PCR, hence the concept of real time. The greater the amount of fluorescence produced with the reaction, the greater the amount of product. A larger amount of product indicates a larger amount of starting material.

qPCR Amplification Cycles. Each of the forty cycles of qPCR amplification are composed of three separate temperatures: separation, annealing, and elongation. The separation temperature (95°C) is high enough to break the hydrogen bond that hold the DNA together, thus separating the double stranded DNA into single strands. The annealing temperature is optimized based on the melting temperature of the primers. The proper melting temperature allows the primers to anneal to the target DNA sequence at the correct position. Finally, the elongation temperature (72°C) is the optimal temperature at which new DNA is synthesized via the action of the Taq DNA polymerase, a DNA synthesizing enzyme that is able to withstand the heat fluctuations without being denatured or destroyed.

The amplification cycles progress in a sigmoidal curve. The curve consists of four phases: ground, exponential, linear, and plateau (Figure 3-7).

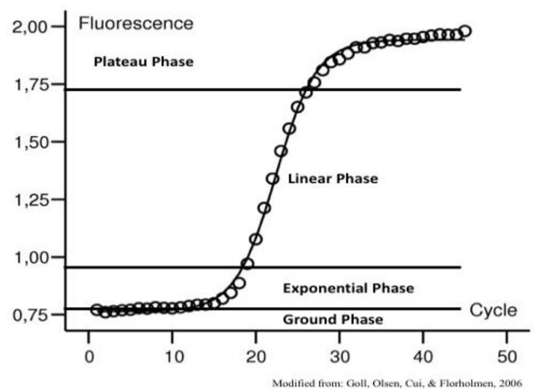

Figure 3-7. The PCR amplification cycles.
A sigmoidal curve that represents the four phases of PCR amplification. The ground phase has no detectable fluorescent amplification; The exponential phase occurs when the fluorescence becomes detectable; During the linear phase, the PCR product doubles with each cycle; Finally, the plateau phase is when the reaction components are exhausted, and the reaction has essentially stopped. Each circle in the diagram represents a cycle of PCR.

No detectable amplification is present in the ground phase, as the fluorescent emission has not yet surpassed the background fluorescence. In the exponential phase, the level of fluorescence has reached a threshold level and is now greater than the background. During the linear phase, the PCR reaction has achieved optimal amplification, with a doubling of PCR product occurring with every reaction cycle. Finally, the plateau, or endpoint phase, is reached when the reaction components become limited and the reaction has essentially stopped. During the plateau phase, the fluorescent intensity of the reaction is no longer useful for data calculation (Wong & Medrano, 2005). The end product of this process is the detection of PCR amplification during each cycle, especially the earlier phases where it is possible to visualize the cycle where the amplification product reached threshold. The number of the threshold cycle is then used to calculate the relative abundance of the sequence of interest.

Considerations When Using qPCR. In real-time qPCR reactions, the differences in cDNA between the samples are corrected for by normalizing the data with a reference gene. An ideal reference gene is one for which expression does not change between samples regardless of experimental condition or time point (Taylor et al., 2010). We used *β-actin* as our reference gene. The protein encoded for by the β-actin gene is present in the structure and motility of all cells and is highly conserved (ubiquitous) across species. Normalization of the data to a reference gene ensures that equivalent amounts of product are being compared across the samples.

Another consideration to take into account are the sources of variability that could affect the reproducibility of the experiment. The two main sources of variability in qPCR reactions are biological variability and technical variability. Biological variability stems from natural differences in levels of gene expression between samples. Technical variability occurs due to differences in the process of conducting the experiment such as differences in pipetting or the quality of the cDNA (Taylor et al, 2010). In an attempt to reduce variability in the results, we designed our qPCR experiment with a combination of both biological and technical replicates. To increase the precision of our qPCR results, the gene expression across all the replicates were averaged. We used at least three biological replicates for each gene and three technical replicates for each biological sample.

Our purpose for using qPCR reactions was the validation of the microarray. The genes that we chose to validate were genes with a documented role in the development or maintenance of pain. After performing the qPCR validation, we wanted to know if the differences shown in the microarray and qPCR gene expression were also present at the protein level. Therefore, our next experiment was to perform Western blot analysis of the proteins translated from our genes of interest.

Western Blot

Western blot analysis is a method of separating and identifying proteins (Mahmood & Yang, 2012). Nurses have used Western blots in research to confirm results of gene expression and mass spectrometry studies that examine HIV-related fatigue (Jensen et al., 2014; Voss et al., 2011), and also to confirm candidate genes established from microarray and qPCR studies examining preclinical preeclampsia (Founds et al., 2015).

The process of Western blot analysis involves the transfer of proteins from a gel onto a membrane, followed by protein detection on the membrane through the use of specific antibodies. The specificity of the antibody allows identification of the protein of interest in a complex protein mixture. Western blots are used to verify the selectivity of antibodies, determine protein modification, determine protein expression changes under altered conditions, and recognize whether or not a particular protein is being expressed in a cell or tissue (Moore, Savage, & Hers, 2013). We used Western blot analysis to confirm the presence of proteins translated from the genes of interest we identified via microarray analysis.

Experimental Workflow. Our Western blot protocol was based on published research (Dorsey et al., 2009; Figure 8). We first homogenized the LH tissue in a lysis buffer designed to simultaneously fragment the cell membrane while protecting proteins from degradation. The homogenate was mixed with sample buffer and boiled for five minutes. This step allows the protein structure to unwind, an action that facilitates the movement of proteins under the influence of an applied electric field (gel electrophoresis).

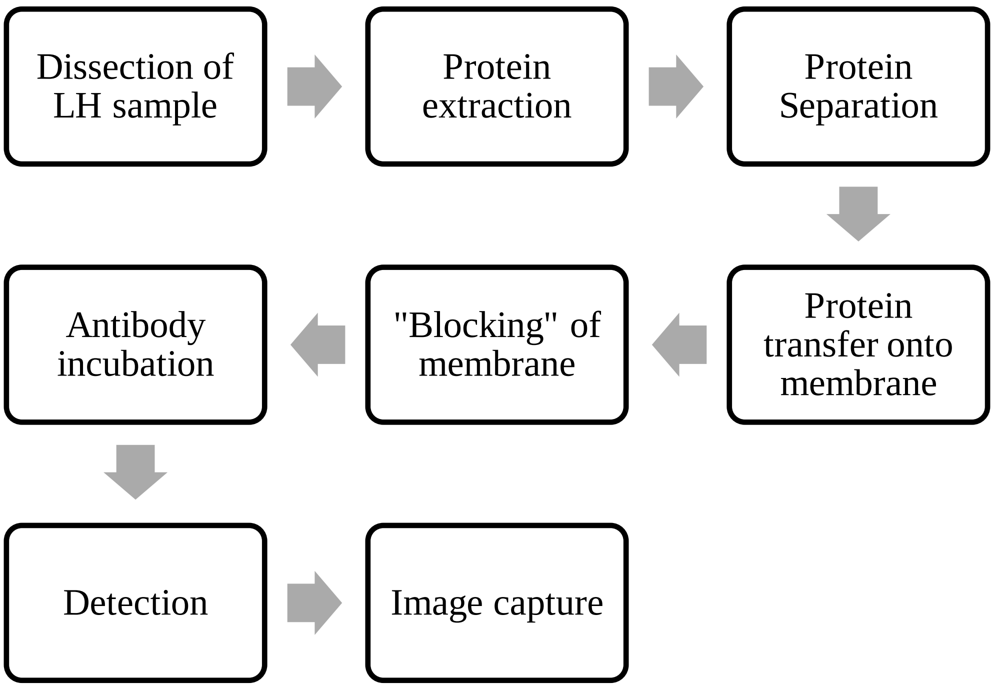

Figure 3-8. Flowchart of Western blot procedure.
After dissecting LH tissue from the mouse brain, the protein was extracted from the tissue. Proteins were then separated according to mass and transferred onto a membrane. The membrane was "blocked" to prevent non-specific binding of proteins. Next, the membrane was incubated in primary antibody to identify the protein of interest followed by the secondary antibody conjugated to a horseradish peroxidase (HRP) detection molecule. The HRP was then excited with chemiluminescence and the image captured.

Protein Separation. The separation of proteins according to mass (kDa) using gel electrophoresis was the next step of our Western blot. Gel electrophoresis uses an electric field to separate the proteins based on their negative charge. Depending on the characteristics of the protein of interest, there are different forms of gels and gel buffers used for the separation. Use of the incorrect gel can lead to inaccurate results. Once the gel was placed in the electrode box and loaded with the protein, the electrode box was filled with a buffer to give the protein a constant charge, allowing the proteins to separate according to size until the sample had reached the foot of the gel. Then, the electrophoresis was stopped, and the gel prepared for protein transfer.

Protein Transfer. The separated proteins were next transferred from the gel onto a membrane. The membrane was laid directly on top of the gel, situated between filter paper, and placed into an electrode box. The entire system was then submerged in a conducting solution, and electric current was applied. The addition of the current forces the proteins to move out of the negatively-charged gel and onto the positively-charged membrane.

After transfer, the membrane was prepared for protein detection by “blocking.” The process of “blocking” involves placing the membrane into a buffer of proteins from either dry milk or Bovine serum albumin to prevent or “block” the binding of non-specific antibodies to the membrane during the remaining steps of the assay. Blocking improves the sensitivity of the primary antibody by reducing background staining without obscuring the specific area of the protein to which the antibody binds, called the epitope. After blocking, the membrane was incubated overnight in primary antibody. The primary antibody binds to the epitope of the protein of interest, then a secondary antibody is used to detect the primary antibody. The secondary antibody is conjugated to a horseradish peroxidase (HRP) enzyme that enables antibody detection (Molnar, 2013).

Primary Antibody. The primary antibody can be either polyclonal or monoclonal (Lipman, Jackson, Trudel, & Weis-Garcia, 2005; Figure 3-9).

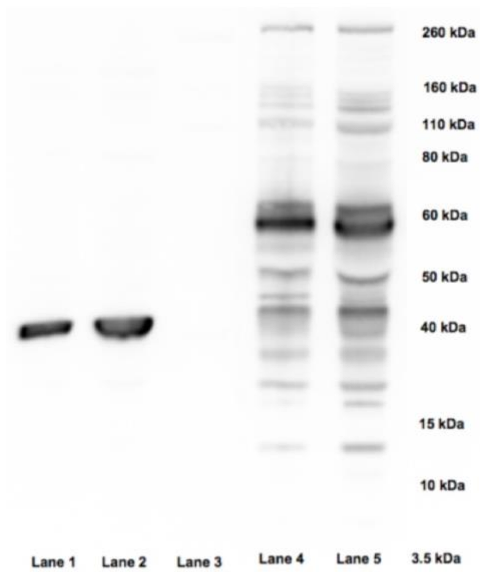

Figure 3-9. Types of primary antibodies.
Primary antibodies can be either monoclonal (Lane 1 and Lane 2) or polyclonal (Lane 4 and Lane 5). Monoclonal antibodies only recognize one epitope on the protein, creating one band on the Western blot. Polyclonal antibodies recognize multiple epitopes on the same protein, creating more than one band on the protein. Generally, the protein of interest is the darkest band. In this case, using the masses from the protein ladder on the right, the darkest band is around 60 kDa, which corresponds with our protein of interest.

A polyclonal antibody recognizes multiple epitopes on the same protein, which can cause cross reactivity with other proteins. Polyclonal antibodies also produce many non-specific antibodies that can create background signal; therefore, more than one band may occur on Western blots that use polyclonal antibodies. Monoclonal antibodies, on the other hand, only recognize one epitope on the protein, hence are less likely to cross-react with other proteins and, due to their specificity, create less background signal. Monoclonal antibodies appear as a single band on Western blots. Because they only produce one band, monoclonal antibodies are preferred for Western blots.

After the membrane was removed from the primary antibody, it was washed to remove the non-specifically bound molecules and reduce the background staining on the membrane

(Moore et al., 2013). Once washes were completed the membrane was placed in a secondary antibody.

Secondary Antibody. An important consideration when choosing a secondary antibody is that the antibody is able to recognize the host species in which the primary antibody was produced. The best secondary antibody is one directed against the species of the primary antibody. For example, if the primary antibody was produced in goat, then the secondary antibody must be an anti-goat antibody produced in a non-goat host. After the primary antibody binds the target protein, the enzyme-linked secondary antibody forms a complex.

Protein Detection. After removing the membrane from the secondary antibody, it was washed, and prepared for protein detection by an enhance chemiluminescence (ECL) substrate. The chemical reaction produced by the addition of ECL to the antibody complex emits light that can be detected on an X-ray film. After the film is developed, the bands that correspond to the protein of interest appear as dark blots. The horizontal location of the band on the blot, corresponds to the mass of the protein (kDa), heavier proteins move more slowly, thus appear closer to the top of the blot, while lighter proteins travel to the foot of the blot more quickly. To assure the correct measurement, we compared our blots to a pre-stained protein ladder. Blots on the pre-stained ladder appear at predetermined masses for comparison.

Quantification. To compare protein expression levels between our samples, we performed quantification of the detected bands. Blots were quantified by scanning JPEG files into ImageJ imaging software (National Institutes of Health, Bethesda, MD, USA) to determine the optical density of each band. To standardize samples for protein loading quantities, after processing, the blots were stripped and re-probed with a primary antibody to either actin or GAPDH followed by incubation with a HRP-conjugated secondary antibody. The membranes were then visualized with ECL. Blots were quantified by scanning JPEG files into ImageJ imaging software (NIH) to determine the optical density of each band.

Advantages, Disadvantages and Considerations of Western Blots. There are both advantages and disadvantages to using Western blot to confirm the presence of protein. The procedure itself is uncomplicated, but it is time consuming, taking up to three days to complete. It is fairly simple to identify large proteins and proteins in high abundance, but smaller proteins or those in a small amount can be hard to detect. To detect the smaller or less abundant proteins, other techniques for protein identification such as enzyme-linked immunosorbent assay or immunoprecipitation may yield better results. Another disadvantage of Western blots is that not all antibodies will work for the assay, and the end result is no bands or many non-specific bands on the blot; for this reason, it is important to test all antibodies prior to using the experimental samples.

Although the experimental protocol for Western blots is quite straightforward, there are some considerations to take into account during each step of the protocol that could otherwise create problems with the assay. Depending on the characteristics of the protein of interest, there are different gels and gel buffers from which to choose for optimal results. The two most common gel types are Tris-glycine and Bis-Tris based gels. Tris-glycine gels are better for detecting subtle protein band shifts or subtle differences in molecular weight. On the other hand, results using Bis-Tris gels have sharper bands and better resolution (Moore et al., 2013). The important considerations in buffer selection are pH and ion concentration as these factors have an effect on power requirements for the electrophoresis to separate the proteins.

It is also important to know how much of the protein sample is recommended to use with each gel. Too much or too little protein could bring about inaccurate results. For example, it is helpful to know how much protein is recommended for the gel type, because if too much is used, the protein will not be able to separate. On the other hand, if there is a small amount of the protein of interest in the sample protein, then enough protein must be injected into the gel, or otherwise the protein will not be detectable., Another consideration involves the choice of membrane type for protein transfer, nitrocellulose or polyvinylidene (PVDF). The binding capacity and resilience of the PVDF membrane is higher, but also requires more rigorous blocking and may have more background signal (Moore et al., 2013). Proper protein transfer also relies on absolute contact between the gel and the membrane. If air bubbles are allowed between the gel and the membrane, the air bubble will insulate the gel and block the transfer of protein (Mahmood & Yang, 2012; Moore et al., 2013).

Other considerations for Western blotting involve testing the antibody in tissue that is known to have the protein of interest, especially if the experimental sample, like LH tissue is in a small quantity. In our experiment, we tested each antibody in whole brain tissue. The reason we used whole brain tissue was to ensure that the antibody detected protein in the brain and to test different amounts of the antibody to find the optimal concentration. Another test that is important to consider for optimal results is a no primary antibody control blot that contains the secondary antibody, but no primary antibody. This control is done to ensure that the secondary antibody only conjugates with the primary antibody and not to any of the epitopes on the protein of interest.

Conclusion

The preceding case study serves as an exemplar of the use of genomic technology in nursing research. In order to provide the highest standard of precision healthcare to patients, nurses should understand the rationale and science behind the methods involved in genomic research. As part of understanding the rationale and science, it is important to know where in the technology errors can occur, how these errors might affect results, and how to avoid these errors. There were many lessons learned in the process of conducting this research study. For instance, to ensure optimal experimental conditions, it was necessary to practice all procedures prior to conducting the experiments with the experimental samples. There are also many considerations to take into account with each technology, and overlooking these issues can have a detrimental effect on the experimental outcome. Through performing her dissertation research, the author not only learned about the effect of gene expression in the LH on the development of allodynia in mice post cisplatin treatment, but also about the precision and meticulous thought that is necessary for performing experiments and understanding omic technology.

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CHAPTER IV

TRANSCRIPTOMIC PROFILE OF THE LATERAL HYPOTHALAMUS IN THE DEVELOPMENT OF PAINFUL CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY BEFORE AND AFTER CISPLATIN TREATMENT IN MICE

Abstract

The lateral hypothalamus (LH) is part of a descending system that modulates pain in the spinal cord dorsal horn. The chemotherapeutic drug cisplatin promotes abnormal pain signaling that may disrupt the genome of LH cells, contributing to the development of painful chemotherapy-induced peripheral neuropathy (P-CIPN). Little is known about how LH cells facilitate P-CIPN. The purpose of this study was to investigate the LH transcriptome of mice to gain insight into the underlying mechanisms of P-CIPN development. Two inbred strains of mice, A/J and C57BL/6J, with differential sensitivity to P-CIPN were examined to evaluate the contribution of the LH transcriptional profiles after four-weeks of bi-weekly treatment with 4 mg/kg of cisplatin. We demonstrated that the A/J strain presented with less severe mechanical hypersensitivity to von Frey filaments than the C57BL/6J strain. Analysis of microarray data showed very few gene expression changes within each strain after cisplatin treatment, but we discovered 1311 differentially expressed genes (DEG) between the strains prior to treatment. Quantitative real-time polymerase chain reaction verified seven DEGs that code for proteins with documented function in pain development. Western blot confirmed two of the proteins. The results of our pre-clinical study suggested that the gene expression profile prior to cisplatin treatment may predispose the development of P-CIPN in mice. These pre-clinical findings may ultimately guide

precision health by using gene expression profiles to predict patients at risk for P-CIPN and identify patients who will benefit from preventative measures.

Introduction

Advances in early detection and treatment of cancer have given rise to almost 14.5 million cancer survivors in the United States (Siegel et al., 2012), a number projected to reach over 19 million by the year 2024 (National Cancer Institute, 2017). For this reason, there is an increasing demand for research devoted to the prevention or amelioration of unwanted side effects of cancer treatment. One of the most debilitating and dose limiting side effects is chemotherapy-induced peripheral neuropathy (CIPN) which occurs in 30 - 70% of individuals who receive neurotoxic chemotherapy (e.g. platinums, vinca alkaloids, bortezomib, and taxanes; Argyriou, Bruna, Marmiroli & Cavaletti, 2012; Cavaletti et al., 2013; Hershman et al., 2014; Kautio, Haanpaa, Kautiainen, Kalso, & Saarto, 2011; Mols, Beijers, Vreugdenhil & van de Poll-Franse, 2014, Seretny et al., 2014). CIPN can manifest with a number of impairments in the sensory, motor, and autonomic nervous systems. Muscle atrophy and weakness may result from motor neuron damage, and autonomic nerve injury can cause constipation, urinary retention, changes in blood pressure, and sexual dysfunction (Smith et al., 2014). Sensory manifestations include burning, numbness, and tingling in extremities; decreased continuous and vibratory sensations; diminished proprioception; and neuropathic pain (Saif & Reardon, 2005; Smith et al., 2014). Painful CIPN (P-CIPN) is reported in up to 40% of patients with CIPN (Kautio et al., 2011; Knoerl et al., 2017; Ventzel, Jenson, Jenson, Jenson, & Finnerup, 2016). P-CIPN can become chronic and persist for months to years after chemotherapy. P-CIPN can be seriously debilitating, causing functional status impairment, decreased workplace productivity, and an overall decrease in quality of life (Mols et al., 2014; Travis et al., 2014). Despite the known negative effects of P-CIPN on quality of life, few treatments and no preventative measures exist (Hershman et al., 2014).

Cisplatin is a first-line chemotherapy drug used in treatment of cancers such as testicular, lung, cervical, ovarian, bladder, head/neck, and endometrium (Allen 1991; Seaver, Greenberg, & Mehnert, 1994; Manji 2011). Cisplatin therapy is associated with the development of CIPN in more than half of patients who receive a total cumulative dose ranging from 225 – 500 mg/m^2 (Argyriou et al., 2012; Argyriou, Kyritsis, Makatsoris & Kalofonos, 2014). Known risk factors include prior or simultaneous administration of taxanes, single and cumulative cisplatin dose levels, and pre-existing peripheral neuropathy (Argyriou et al., 2014). The mechanism of toxicity is the result of platinum-DNA binding in the dorsal root ganglia (DRG) of the nerve cell bodies (McDonald et al., 2004).

When the DRG of sensory neurons are exposed to toxic levels of cisplatin, disruption can occur in the sensory pathways that are responsible for the normal modulation of pain. The spinohypothalamic ascending sensory pathway transmits the impulse from the spinal cord to the brain via the hypothalamus (Burstein, Cliffer, & Giesler, 1990), and descending pathways modulate nociception (the processing of a noxious, or painful, stimuli) at the level of the spinal cord dorsal horn via signals that travel from higher cortical levels of the brain through the lateral hypothalamus (LH) to the spinal cord (Holden & Naleway, 2001; Holden, Van Poppel & Thomas, 2002; Holden et al., 2014). Stimulation of the LH promotes the release of norepinephrine in the dorsal horn that attenuates or blocks incoming nociceptive action potentials. This system is tonically active in neuropathic pain releasing norepinephrine that can bind both pain-promoting alpha$_1$- (pronociceptive) and pain-inhibiting alpha$_2$- (antinociceptive) adrenoceptors (Wagner et al., 2016). The net effect is antinociception, but the opposing pronociceptive activity likely attenuates this antinociception.

The International Association for the Study of Pain (IASP) describes central sensitization as increased responsiveness of nociceptive neurons in the central nervous system to normal or subthreshold afferent input (1994). One of the characteristic features of central sensitization is the loss of the ability of dorsal horn neurons to respond selectively to noxious stimuli, leading to a non-noxious stimulus becoming noxious (allodynia), which is a distinguishing feature of neuropathic pain (Romanelli & Esposito, 2004). Neuropathic pain has also been implicated in changes within the brain. There is evidence of reorganization of cortical circuits. Cell-type specific changes significant in the development of neuropathic pain are noted in the somatosensory cortex of mice post sciatic nerve injury (Cichon, Blanck, Gan, & Yang, 2017). Following the development of neuropathic pain, male rats show increased levels of *c-fos*, which is expressed after a painful stimulus, in the frontal cortex, thalamus, and periaqueductal gray (Narita et al., 2003). Although it is not known whether LH neurons are altered in conditions of P-CIPN after cisplatin treatment, we predicted that chronic nociceptive input into the LH could alter its transcriptome.

The cell bodies of the peripheral nerve fibers (A-α and A-β touch fibers; A-δ and C pain fibers) are located in the DRG. Cisplatin binds to DNA of peripheral nerve fibers in the DRG and induces nuclear and mitochondrial DNA damage that causes an alteration in nerve fiber function (McDonald et al., 2005; Podratz et al., 2011). The altered function can modify the physiology, chemistry, and DNA of dorsal horn neurons that the peripheral nerve fibers synapse with as part of central sensitization (Romanelli & Esposito, 2004). The LH receives nociceptive information from the dorsal horn via the ascending spinohypothalamic tract (Almeida, Roizenblatt, & Tufik, 2004; Burstein, Cliffer, & Giesler, 1990), making it possible that cisplatin related DRG injury can produce central sensitization of LH neurons in a manner similar to central sensitization of the spinal cord neurons, and these changes may contribute to gene expression changes in the LH that alter descending pain modulation.

In this study, we examined the transcriptome of the LH in C57BL/6J and A/J mice before and after cisplatin treatment to detect gene expression changes associated with the development of P-CIPN. Using the nocifensive behavior of paw withdrawal from a mechanical stimulus to model the development of P-CIPN, the aim of our study was to determine target genes that may be part of a gene expression profile associated with the development of P-CIPN.

Methods

Animals. We used adult female mice: C57BL6/J, A/J (n = 14 per strain; 15–25 g; The Jackson Laboratory, Bar Harbor, ME). All mice were housed on a 12:12 hour light/dark cycle with food and water available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore School of Medicine, and conducted in accordance with the IASP guidelines for investigation of pain in animals (Zimmerman 1983). In accordance with these guidelines, we used the minimum numbers of animals to meet the rigor necessary for this series of experiments. On testing days, the mice were weighed to detect changes in food and water consumption. Any mouse that demonstrated signs of debilitation or a >20% weight loss from baseline was euthanized. All mice were euthanized upon completion of experiments. The mouse brains were then dissected for LH tissue extraction.

Drug Administration. Cisplatin (MWI Veterinary Supply; Boise, ID) was diluted in 0.9% saline to a concentration of 20 mg/ml and administered to mice via intraperitoneal injection of 4 mg/kg twice a week for eight doses. The vehicle groups were injected with 0.9% saline alone. Volumes of saline were adjusted to the weight of each mouse and injected via the same route.

Nocifensive Behavioral Testing. Mechanical allodynia was measured via paw withdrawal from a mechanical stimulus (Dorsey et al., 2009). A series of von Frey filaments (Touch Test Sensory Evaluator Kit, myNeurolab.com, St. Louis, MO), with bending forces ranging from 0.04g to 1.40g, were used to deliver the stimuli. Three cohorts of mice (cisplatin group n = 6, vehicle group n = 6, and naïve group n = 6) were tested. Naïve mice were tested before drug administration to determine a threshold for paw withdrawal, defined as the fiber with the smallest bending force that elicited three aversive responses (paw withdrawal) out of five trials. Allodynia was determined to be present if the response threshold shifted to the left, such that a previously non-noxious fiber with a bending force less than the naïve threshold fiber elicited three aversive responses out of five trials. All groups were tested prior to treatment and then once per week throughout administration to observe changes in behavioral responses over time.

To perform the testing, the mice were placed in individual Plexiglas cubicles (8.5 cm in length x 4 cm in height x 4 cm in width) on an elevated wire mesh platform and allowed to acclimate for approximately 1 hour. Each filament was applied to the hind paw until the filament was just bent and held in place for 5 seconds, or until the mouse withdrew its paw. Each filament was tested five times on each hind paw. The stimuli were applied to the plantar surface of the hind paw, starting with the 0.4g filament. If the 0.4g filament elicited three positive responses out of five trials, then the mouse was tested moving downward through the filament series toward 0.04g until the filament with the smallest bending force to elicit three positive responses was identified and recorded as the threshold fiber. If the 0.4g filament did not elicit three positive responses, then the mouse was tested moving upward through the series toward 1.4g until the filament with the smallest bending force to elicit three positive responses was identified and recorded as the threshold fiber. The observer was blind to condition in all testing situations.

Total RNA Extraction. Separate cohorts of mice were used for microarray and qPCR studies. Mice used for the microarray study were euthanized 24 hours after the eighth dose of cisplatin. This time point was chosen because it was best able to induce the behavioral (Carozzi et al., 2015) as well as the physiological and morphological (Carozzi et al., 2010; Carozzi et al., 2015) changes seen with cisplatin treatment. Because we ultimately examined gene expression differences prior to cisplatin treatment, naïve animals were used for qPCR. To maintain consistency in the conditions between the two experiments, the qPCR cohort was age-matched to the animals used for microarray.

To perform the tissue harvest, the mice were euthanized by cervical dislocation followed by rapid removal of the brains, which were immediately flash frozen on dry ice. Total RNA was extracted from cisplatin-treated, vehicle-treated, and naïve mice using a standard TRIzol®-based extraction method (Invitrogen™ Life Technologies™, Grand Island, NY, USA) as previously described (Dorsey et al., 2009). Briefly, whole brains were sliced along the coronal plane within the area defined as the LH (Paxinos & Franklin, 2008). The first slice was along the lambda suture, the next slice was 2mm rostral to lambda, and the third was 1.5 mm rostral to the previous cut, to remove a 1.5 mm brain section. From this section, tissue was punched out 0.5 mm above the lower crevasses on both the right and left side of the brain using a leveled off 20-gauge syringe. LH tissue was then placed in TRIzol® and homogenized into a solution using the Next Advance Bullet Blender® with Zirconium Oxide 0.5 mm beads. Chloroform was added, and then the mixture was separated into phases via centrifugation. Total RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol, and then re-suspended in 20 µL diethylpyrocarbonate (DEPC) water (Invitrogen™ Life Technologies™, Grand Island, NY, USA). Concentration and quality of RNA was determined via spectrophotometer (NanoDrop1000, Thermo Scientific, Wilmington, DE, USA). Samples with a 260/280 ratio of approximately 2.0 were considered sufficient for use in subsequent gene expression tests. The samples were stored at -80ºC until further analysis.

RNA Labeling and Microarray Processing. All microarrays were processed by one person in the same laboratory following a standardized lab protocol to minimize non-biological technical bias. We used the GeneChip® Mouse Genome 430 2.0 array from Affymetrix (Carlsbad, CA, USA), which provides coverage for over 34,000 mouse genes. Total RNA (~200 ng) was used to prepare double-stranded cDNA. The quality and quantity of RNA was assessed on a bioanalyzer 2100 using Agilent Technologies RNA nano chips. Samples with a 260/280 ratio of approximately 2.0 and two sharp peaks that corresponded to the 18S and 28S on the RNA gel were considered of sufficient purity to be used in the microarray analysis. Next, double-stranded cDNA was used as a template in an *in vitro* transcription reaction to prepare biotinylated cRNA. The biotinylated target was fragmented and hybridized to the probes contained on the expression arrays. After hybridization, the arrays were washed and stained in the Affymetrix fluidics station and scanned in the 3000 7G scanner. Intensities of probe hybridization were detected by laser scan of the array. The .CEL files were generated by Affymetrix GeneChip software (MAS5).

Bioinformatics. Microarray analysis was conducted using R software packages developed by Bioconductor (www.bioconductor.org). To check the quality of the arrays and to identify potential outlier arrays, the .CEL files were analyzed using clustering, Principal

Component Analysis, and the affyQCReport package (v.1.48.0). The Robust Multi-Array Average (RMA) method (Irizarry et al., 2003) implemented in the Bioconductor affy (v.1.48.0) package was used to background correct the raw intensities, normalize them across arrays, and then summarize them to obtain expression values at the probe level for each array. The normalized microarray data were analyzed by both one-way and two-way ANOVA using the Limma R package (v.1.48.0; Smyth 2004) to determine the effects of both treatment and strain on the development of allodynia in mice. To correct for error inflation due to multiple statistical testing, the raw p-value was adjusted into the False Discovery Rate (FDR) using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). We used an FDR value at the level of ≤ 0.10 and a gene expression fold change of $\pm$ 1.5 as cutoff points for significance. To identify canonical pathways and functions altered by cisplatin treatment we analyzed the list of differentially expressed genes using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). Raw and processed microarray data will be deposited into the Gene-Expression Omnibus (GEO) database upon publication, accession number (pending).

qPCR Verification of Identified Target Genes. Target genes identified from the microarray analysis were validated using a quantitative real-time qPCR approach. This technique uses PCR to detect, amplify, and quantify the absolute number of copies of a specific DNA sequence in the experimental sample in relation to a normalized DNA sample. Naïve mice (n = 5) from each strain were sacrificed and RNA was extracted as described above. Harvested total RNA was reverse transcribed using Superscript® III reverse transcriptase and oligo (dT) primers (Invitrogen). Forty cycles of qPCR were performed using the Lightcycler 480 SYBR Green I Master Mix (Roche Life Science, Indianapolis, IN). Fluorescence dye was used to observe double-stranded DNA. Plotting fluorescence as a function of temperature, the thermal cycler heats through the dissociation temperature of the DNA, producing a DNA melting curve. The shape and position of the curve was used to differentiate between the amplified DNA sequences. The relative abundance of each sequence was computed using Roche Lightcycler 480 Relative Quantification software (Roche Life Science). The primer sequences used to amplify each gene are listed in Table 4-1 (Integrated DNA Technologies). The β-actin gene was used as a reference gene.

Table 4-1. Nucleotide sequences of primers for qPCR.

Gene	Forward Primer 5’ → 3’	Reverse Primer 5’ → 3’
Beta-actin	5’ CCC GCG AGC ACA GCT TCT TT 3’	5’ GCC CAC GAT GGA GGG GAA TAC 3’
Calcium/calmodulin-dependent protein kinase IV (*CAMKIV*)	5’ CTG GAT CGA CGG CTC TAA CC 3’	5’ GAG CAT AGG GCT TCT GGG TC 3’
Protein kinase C, delta (*PRKCD*)	5’ TAT TCG AGA GGG ACC CTG ACA 3’	5’ AAC TCT GGG TCA AAG TTG CTG 3’
Brain derived neurotrophic factor (*BDNF*)	5’ CTT CCT GCA TCT GTT GGG GA 3’	5’ ACA CCT GGG TAG GCC AAG TT 3’
Glutamate receptor, metabotropic 7 (*GRM7*)	5’ CCT GCC TGC TTC CTA TCT CTG 3’	5’ CCG GGT GGG ACT TGA ATC TC 3’
Potassium voltage-gated channel, subfamily Q, member 2 (*KCNQ2*)	5’ TCC CTG GGG AGG ACA TCG 3’	5’ GTG TCC AGC CGA GTA CTG TT 3’
Glial fibrillary acidic protein (*GFAP*)	5’ TCT CCA ACC TCC AGA TCC GA 3’	5’ TGG TGA GCC TGT ATT GGG AC 3’
cAMP response element binding (CREB) binding protein (*CREBBP/CBP*)	5’ ACA AGC GAA ACC AAC AAA CCA T 3’	5’ TGG AAC TGG GGT CTA TGG GA 3’
Glutamate receptor, ionotropic, AMPA2 (alpha 2) (*GRIA2*)	5’ GGG AAG TAA GGA AAA GAC CAG TG 3’	5’ CTG GGA ATT CTG CGA GGA AGA 3’
Interleukin 15 (*IL15*)	5’ TTC TCT GCG CCC AAA AGA CT 3’	5’ GGT GGA TTC CCT GAC CTC T 3’

Western Blot Analysis. To determine whether changes seen in each of the target genes resulted in changes in the level of protein product expressed, Western blot analysis was performed. Euthanization of mice and removal of the LH followed the same procedure described above. The LH tissue was then mechanically homogenized in lysis buffer containing phosphatase and protease inhibitors (Tris-buffered saline plus 10% glycerol, 0.1% NP-40, phosphatase and protease pellets [Roche Life Science]; 100 mg tissue to 1 mL lysate ratio). Sodium dodecyl sulfate (SDS) solubilized tissue was then incubated at 100°C for 5 minutes with an equal amount of 2-mercaptoethanol to reduce the intra- and inter- molecular disulfide bonds, fractionated on 4-12% NuPAGE bis-tris gels (Invitrogen) and transferred to a nitrocellulose membrane. After membranes were placed in nonfat dried milk to reduce non-specific antibody binding, membranes were incubated overnight at 4°C with a primary antibody to the protein of interest followed by incubation with horseradish peroxidase-conjugated secondary antibody (Table 4-2).

Table 4-2. Antibodies used for Western blot.

Protein	Primary Antibody	Secondary Antibody
Beta-actin	Cell Signaling Technology Rabbit Monoclonal (1:1000)	Cell Signaling Technology Anti-rabbit IgG HRP-linked AB (1:2000)
Calcium/calmodulin-dependent protein kinase IV (CAMKIV)	Cell Signaling Technology Rabbit Polyclonal (1:500)	Cell Signaling Technology Anti-rabbit IgG HRP-linked AB (1:2000)
Protein kinase C, delta (PRKCD)	Cell Signaling Technology Rabbit Monoclonal (1:1000)	Cell Signaling Technology Anti-rabbit IgG HRP-linked AB (1:2000)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Cell Signaling Technology Rabbit Monoclonal (1:500)	Cell Signaling Technology Anti-rabbit IgG HRP-linked AB (1:2000)

The membranes were then visualized with chemiluminescence (ThermoFisher Scientific). Blots were quantified by scanning JPEG files into ImageJ imaging software (National Institutes of Health, Bethesda, MD, USA) to determine the optical density of each band. To standardize samples for protein loading quantities, after processing, the blots were stripped and re-probed with a primary antibody to either actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Table 4-

2). The membranes were then visualized with chemiluminescence (ThermoFisher Scientific). Blots were quantified by scanning JPEG files into ImageJ imaging software (NIH) to determine the optical density of each band.

Statistical Analyses. The behavioral data, expressed as mean gram force ± SEM, were analyzed with two-way analysis of variance (ANOVA), using strain and treatment as factors (independent variables).

Results

Cisplatin Produced Mechanical Allodynia in C57BL/6J Mice but not A/J Mice. C57BL/6J mice treated with cisplatin developed robust mechanical allodynia after one week of cisplatin administration with a significant decrease in paw withdrawal threshold ($F_{(2,60)} = 44.87$; $p < 0.001$) that persisted throughout four weeks of testing when compared to naïve and saline-vehicle treated mice (Figure 4-1A). A/J mice treated with cisplatin showed no significant difference in paw withdrawal threshold between the three cohorts of A/J mice (Figure 4-1B), in the first week of treatment. Unfortunately, due to a > 20% total body weight loss, the cisplatin cohort of A/J mice had to be euthanized after the first week. These results mean that the same dose of cisplatin led to allodynia development in one strain of mouse, but not in the other.

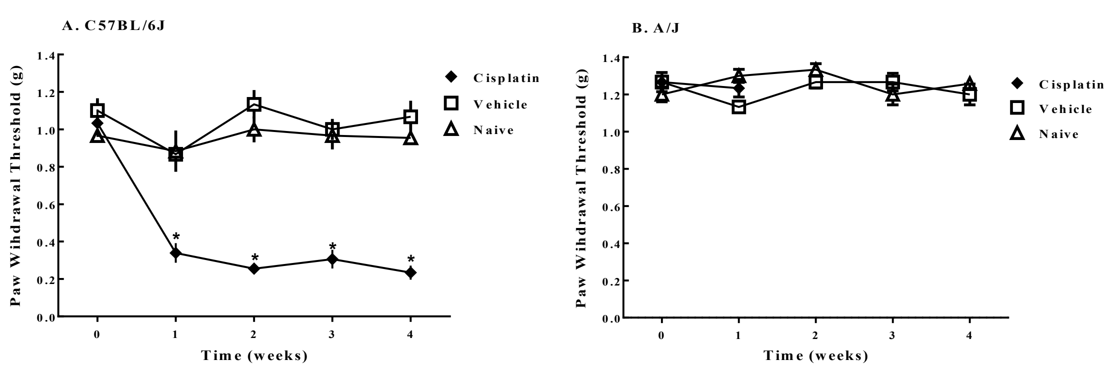

Figure 4-1. Effect of cisplatin administration on paw withdrawal threshold.

Following a baseline response threshold, mice we given either a normal saline vehicle (☐) , or cisplatin (◆), or received no treatment (△; naïve) at time 0 (baseline). C57BL/6J mice (A) that received cisplatin showed significant mechanical allodynia versus normal saline and naïve controls (* $p < 0.001$). Due to significant weight loss, A/J mice (B) that received cisplatin were euthanized after the first week of treatment, but at one week (Time 1), there was no difference in response threshold between the three cohorts of mice (p = NS). Mean withdrawal threshold ± SEM is plotted on the ordinate as a function of time; $n = 6$ mice per group.

Microarray Analysis Demonstrated Gene Expression Differences in Naïve Mice. We examined the results of lateral hypothalamus-derived mRNA gene expression microarray using multivariate analysis using both strain and treatment as factors and found that treatment had no effect on gene expression. We then conducted a reduced analysis that only considered strain differences in untreated (naïve) animals. We found 1311 differential LH-expressed genes (598 up-regulated and 713 down-regulated) between the two strains in the naïve condition that met both a false discover rate (FDR; ≤ 0.10) and gene expression fold-change (± 1.5) criteria.

Using Ingenuity Pathway Analysis (IPA) we identified the top five differentially-enriched canonical, or known, biological signaling pathways (Table 4-3) that distinguish the C57BL/6J and A/J strains of mice.

Table 4-3. The top five canonical biological signaling pathways

Canonical Pathway	Up-regulated	Down-Regulated	Total genes in pathway
1. Cleavage and Polyadenylation of Pre-mRNA	4	1	12
2. Synaptic Long-Term Potentiation	17	8	115
3. CREB Signaling in Neurons	27	12	178
4. BMP Signaling Pathway	13	3	71
5. Neuropathic Pain Signaling in Dorsal Horn Neurons	7	16	109

Each of the pathways is related to neuronal function and can potentially affect the development of CIPN. We next identified eight genes (*PRKCD, KCNQ2*, *CAMKIV*, *CREBBP*, *GFAP*, *IL15*, *GRIA2*, *GRM7*) from the pathway analysis that met the criteria for FDR and fold change, plus have a documented role in neuropathic pain, for further study. In addition, a ninth gene that only met the fold-change criteria (*BDNF*) was recognized based on its documented role in neuropathic pain (Table 4-4; Appendix). The *CAMKIV* gene was present in four of the top five canonical pathways identified via IPA (CREB signaling in neurons, synaptic long-term potentiation, BMP signaling, and neuropathic pain signaling in dorsal horn neurons) *GRIA2*, *GRM7*, and *PRKCD* were present in three of the top five pathways (CREB signaling in neurons, synaptic long-term potentiation, and neuropathic pain signaling in dorsal horn neurons). In addition, five genes (*CAMKIV*, *CREBBP*, *GRIA2*, *GRM7*, *PRKCD*) were present in two top pathways (CREB signaling in neurons and synaptic long-term potentiation). Other relevant genes from the IPA core analysis were included for further analysis. *BDNF* and *KCNQ2* were both expressed in the neuropathic pain signaling in dorsal horn neurons canonical pathway; *IL15* was included due to its role in inflammatory effects; *GFAP* was the top down-regulated gene in the dataset.

Gene Expression Validation with qPCR. We used LH tissue of naïve A/J and C57BL/6J mice that was from a separate set of animals than the tissue used in the microarray for qPCR experiments in order to validate mRNA transcripts from the target genes. Seven of the genes (*IL15*, *CAMKIV*, *GRIA2*, *GRM7*, *KCNQ2*, *GFAP*, and *BDNF*) had gene expression changes in the same direction as the microarray results, but at different magnitudes of fold change. The remaining two genes (*CREBBP* and *PRKCD*) showed gene expression changes in the opposite direction from the microarray (Figure 2A, B). Student's t-test was used to validate the gene expression differences in *IL15* (p = 0.041), *CAMKIV* (p = 0.011), and GFAP (p < 0.001).

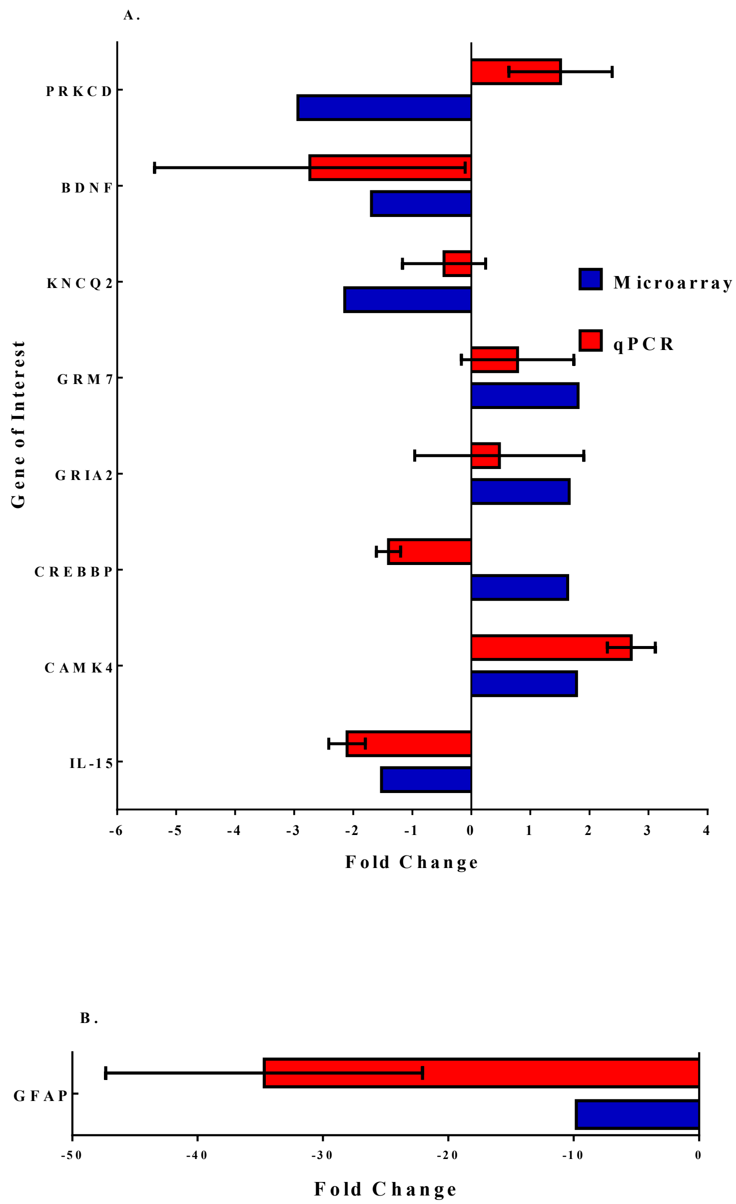

Figure 4-2. Validation of gene expression fold change using qPCR.
LH tissue from naïve mice was used to verify expression levels of genes identified from the microarray. Results are reported as linear fold change in naïve A/J mice as compared to naïve C57BL/6J mice. Most fold changes ranges for -6 to 3 (A), but the fold change for *GFAP* was much larger (B); n = 3-5 per group for each gene.

Western Blot Analysis to Quantify Protein Expression. To determine whether the differences in gene expression were also present in the transcribed protein product, Western blot analyses were performed. Analysis of protein lysate from LH tissue of both C57BL/6J and A/J naïve mice identified the targeted protein products for two genes. The protein product of PRKCD was up-regulated 95% in the A/J mice as compared to the C57BL/6J mice (Figure 3A, B), and the protein product of CAMKIV was down-regulated 12% in the A/J as compared to the C57BL/6J mice (Figure 4A, B).

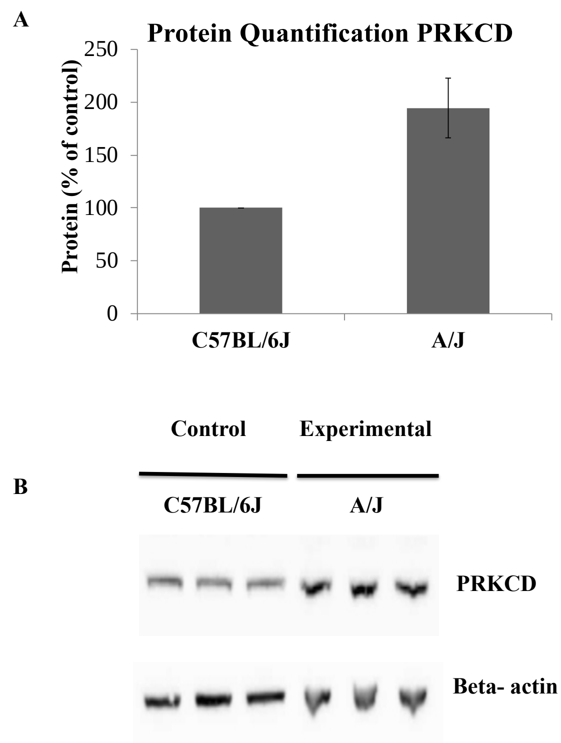

Figure 4-3. Western blot analysis of PRKCD.
Analysis of protein lysate from LH tissue of both C57BL/6J and A/J mice identified target protein product for PRKCD (A). The protein product of PRKCD was up-regulated 95% in the A/J mice as compared to the C5BL/6J mice (B).

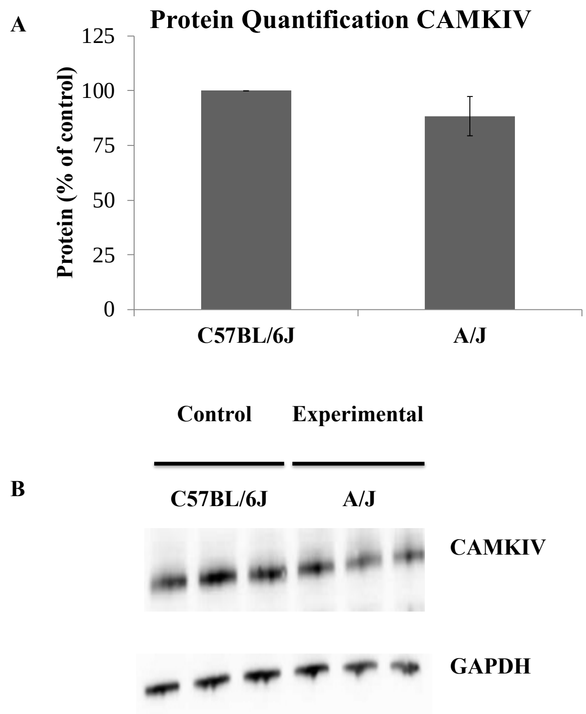

Figure 4-4. Western blot analysis of CAMKIV.
Analysis of protein lysate from LH tissue of both C57BL/6J and A/J mice identified target protein product for CAMKIV (A). The protein product of CAMKIV was down-regulated 12% in the A/J as compared to the C57BL/6J mice (B).

Discussion

The current study was designed using microarray technology to reveal LH-derived genes involved in promoting the development of P-CIPN following chemotherapy treatment with the drug cisplatin. Using an unbiased whole transcriptome approach, we uncovered novel results in explaining differential P-CIPN development between the C57BL/6J and A/J strains of mice. A limited number of genes were differentially expressed after cisplatin treatment, but hundreds of genes were differentially expressed between C57BL/6J and A/J mice that received no treatment. Importantly, the lack of gene expression changes after cisplatin treatment coupled with the differential allodynia development suggested that, in this study, gene expression of the mice **prior to** cisplatin treatment may be a crucial factor in the development of P-CIPN. To further support the idea that P-CIPN development is affected by gene expression prior to cisplatin treatment, using Ingenuity Pathway Analysis to identify canonical pathways and functions, we discovered that one of the top canonical pathways altered between untreated C57BL/6J and A/J mice was “Neuropathic Pain Signaling in Dorsal Horn Neurons.”

Typically, rodent studies investigating the development of CIPN examine changes in tissue associated with the site of injury, such as the DRG (Marmiroli et al., 2017) or site of central sensitization, such as the spinal cord dorsal horn (Makker, 2017). Instead, our study examined the transcriptional profile of the LH, in relation to allodynia development, a sign of P-CIPN. As the location of the third order neuron in the spinohypothalamic ascending pathway, the LH is a unique and valuable area of study. CIPN development is attributed to cisplatin accumulation in the DRG (Gill & Windebank, 1998; McDonald et al., 2004), the anatomical location of the cell body of the first order neuron. The purpose of the first order neuron is the transmission of sensory information from the periphery to the spinal cord dorsal horn, where the first order neuron then synapses with a second order neuron. The axon of the second order neuron decussates to the contralateral side of the spinal cord and then transmits the ascending sensory information via the spinohypothalamic tract to the LH (Burstein et al., 1990.) The cell body of the third order neuron is located in the LH. It could be that the LH, while being part of the transmission of pain, is, as a third-order neuron is too far removed from the initial point of injury to experience gene expression changes after cisplatin treatment.

It was not surprising that we found few gene expression changes in the LH, as cisplatin accumulates in the DRG and does not cross the blood-brain barrier, leaving motor neurons and other central nervous system neurons without direct exposure to toxic levels of cisplatin (Fischer el al., 2001; Gill & Windebank, 1998; McDonald et al., 2004; Seaver et al., 1994). Reanalyzing the dataset to compare untreated C57BL/6J and A/J mice, we discovered baseline gene expression differences that may help explain the difference in the rate of allodynia development between the strains. It was not the intent of our study to determine that a predisposition of gene expression levels is the sole reason for allodynia development. Rather, we propose that baseline gene expression is a factor in the development.

As early as one week after the start of cisplatin treatment, results of nocifensive behavioral testing showed that C57BL/6J mice develop a robust allodynia while A/J mice were not different from naïve mice. This is not surprising as C57BL/6J mice are reported to be more sensitive to cisplatin-related neurotoxicity (Podratz et al., 2016). The same dosing and testing schedules were used for all the mice in this study. The only difference between the cisplatin treated C57BL/6J mice and the cisplatin treated A/J mice was the genetic background of the strain. Our results are similar to results of a recent study exploring the transcriptome of the DRG in C57BL/6J mice after oxaliplatin induced CIPN that shows no significant gene expression changes but a significant effect of oxaliplatin on the development of mechanical allodynia (Marmiroli et al., 2017). This is a surprising result concerning the fact that the DRG is the site of insult in the oxaliplatin-induced CIPN study but lends credibility to the results of our study.

There is an unmet need for the identification of a genetic profile that can help identify patients at risk for CIPN development (Argyriou, Bruna, Genazzani, & Cavaletti, 2017). The development of a genetic profile for those at risk for CIPN should be supported by animal studies that highlight the relevance of the identified genes to improve the understanding of the mechanism of CIPN development (Argyriou et al., 2017). The differentially expressed genes identified in our animal study encode for proteins linked to biological actions such as neuronal development and function, cell signaling, ion channel activity, transcription factor co-activation, identification of astrocytes, and immune cell activation. Research studies investigating the pharmacogenetics of CIPN in human clinical trials have identified an association between platinum-related CIPN development and genes that encode for proteins in ion channel activity, neuronal development and function, cell cycle, DNA repair, cell signaling, and apoptosis pathways (Argyriou, et al., 2017; Johnson et al., 2015). Our animal study identified expression differences in genes encoding for proteins whose functions are linked to mechanisms of clinical CIPN development. Although it should be noted that these clinical studies compare patients who develop CIPN with patients who do not develop CIPN and baseline gene expression is not discussed. It might be that CIPN development in these patients is influenced by baseline expression of genes linked to biological pathways active in CIPN development.

The top pathways from our analysis involve the nervous system and have potential implications in development of P-CIPN; for example, synaptic long-term potentiation, is an increased response to repeated inputs after stimulation and has been thought of as one of the mechanisms underlying the development of central sensitization, of which allodynia is a sign (Baron, Hans, & Dickenson, 2013). CREB signaling in neurons, another top pathway, is implicated in the hyperactivity of dorsal horn neurons after noxious stimulation and is up-regulated in spinal cord injury male rats that have developed neuropathic pain (Crown, Ye, Johnson, Xu, McAdoo, & Hulsebosh, 2006). CREB also has a direct association with increased gene expression in rats exhibiting mechanical allodynia (Crown, Ye, Johnson, Xu, McAdoo, Westlund, & Hulsebosch, 2005).

Most of the genes identified in this study are involved in more than one of the top canonical pathways. The genes *CAMKIV*, *CREBBP*, *GRIA2*, *GRM7*, and *PRKCD* encode for proteins of the “Synaptic Long-Term Potentiation” pathway. The genes *CAMKIV*, *GRIA2*, *GRM7*, *PRKCD*, and *CREBBP* all encode for proteins involved with “CREB Signaling in Neurons.” The genes *CAMKIV*, *GRIA2*, *GRM7*, *PRKCD*, and *BDNF* encode for proteins active in the “Neuropathic Pain Signaling in Dorsal Horn Neurons” pathway. Recognizing that the actions of proteins are not isolated to one pathway is important as we think about symptom science and the biological mechanisms behind symptom development and how it may be that particular genes and biological processes underlie the development of multiple symptoms (McCall et al., 2018).

The microarray analysis showed that there was a 9.78-fold expression difference in *GFAP* between the A/J and C57BL/6J strains of mice, while the difference detected by qPCR was much greater. The *GFAP* difference is interesting because the role of glial cells in neuropathic pain development is an expanding area of research (Machelska & Celik, 2016). Yet, the large difference in gene expression is puzzling. The difference may be due to technical variability instead of biological variability. The distribution of GFAP protein is consistently uneven throughout the brain (Kalman & Hajos, 1989). Although, the LH dissections were all done by the same individual, there could be inequalities in the tissue extraction that account for the difference in expression. An alternative explanation is that the decreased *GFAP* expression in the A/J mice in some way serves as a protection against P-CIPN development.

The involvement of GFAP expression in P-CIPN development implied that not only is the neuron involved in P-CIPN development, but the glia cells that support the neuron are also involved. To confirm this result, further studies should be performed isolating the glial cells from neurons prior to gene expression analysis to ascertain the involvement of each cell type in P-CIPN development.

Only a subset of patients that receive neurotoxic chemotherapy eventually develop P-CIPN. The management of P-CIPN is challenging because not every patient responds to treatment efforts and there are no consistent, reliable, and valid means by which to predict who will develop P-CIPN. At present, there are no preventative measures and only one approved treatment that is not effective for all patients (Smith et al., 2013; Hershmann et al., 2014). Consequently, there is still a need for an evidence-based measure to determine whether an individual is at risk for developing CIPN. A recent review found that, although much effort has been exerted into identifying a genetic profile to identify individuals at risk for CIPN, research results vary widely (Argyriou, et al., 2017). The majority of these studies are genome-wide association studies using a candidate gene approach. This method differs from our pre-clinical study, as we explored the entire LH transcriptome, rather than limiting ourselves to certain candidate genes. This approach allowed us to examine every gene being expressed in the LH at the time of tissue harvest and not a finite list of predetermined genes, thus permitting the discovery of novel gene targets. In terms of platinum-related P-CIPN, clinical studies are targeted more towards the genes that encode proteins involved in ion channel activity, neuronal development and function, as well as apoptosis, and oxidative stress (Argyriou et al., 2017). The genes examined in our study are active in these functions but rather than examining how these genes are changed by chemotherapy, our pre-clinical study found that it may be the expression levels of these genes at baseline that predicts development of P-CIPN in mice. With the current focus on the practice of precision health, our study could be a prime example of a condition best treated when individualized. So that, perhaps, in the future, prior to receiving neurotoxic chemotherapy, each patient will undergo baseline gene expression testing to determine whether or not they will develop P-CIPN.

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CHAPTER V

SUMMARY

As the prevalence of cancer increases, so does the incidence of chemotherapy-induced peripheral neuropathy (CIPN), a common adverse effect of many chemotherapeutic agents used to treat cancer. About 60% of patients receiving the platinum-based chemotherapeutic agent, cisplatin, show evidence of peripheral nerve damage (Argyriou et al., 2012), and 20% cannot complete a full course of therapy due to CIPN (McDonald et al., 2005). CIPN is characterized by symptoms of pain, burning, numbness, and tingling in the extremities (Saif & Reardon, 2005; Smith et al., 2014) that can persist from months to years after completion of treatment (Argyriou et al., 2012; Miltenburg & Boogerd, 2014). CIPN is reported as painful in up to 58% of patients (Kautio, et al., 2011; Loprinzi et al., 2011). It is refractory to traditional treatments and can lead to dose reduction, delay, or early termination of a potentially successful chemotherapy treatment. In spite of tremendous research efforts, there is only one recommended treatment and no preventative measures (Hershman et al., 2014). It is difficult to predict which patients will develop CIPN or the severity of CIPN a patient will experience. Thus, the purpose of this preclinical research was to use microarray technology and pathway analysis to detect gene expression changes in the transcriptome of the lateral hypothalamus (LH) of different strains of mice before and after cisplatin treatment in an animal model of CIPN. Expression changes of select genes were then verified with quantitative real-time polymerase chain reaction (qPCR) and protein translation was confirmed via Western blots.

Results

Sample. Our study used an animal model of cisplatin-induced allodynia, starting out with five inbred (C57BL/6J, BALB/cJ, DBA/2J, A/J, FVB/NJ; Jackson Laboratories) and one outbred (CD1; Charles River Laboratories) strains of mice. A total of 136 mice were used. Over 95% of the mouse genome is similar to that of humans (Hardouin & Nagy, 2000), making research with mice applicable to human disease. Each mouse strain was chosen based on previous work showing the differences across mouse strains in response to treatment with the chemotherapeutic taxane paclitaxel (Smith, Crager, & Mogil, 2004). The chosen inbred strains included mice that had been sibling mated for at least 20 generations, making them essentially genetically identical (Smith et al., 2004). The outbred strain contained a larger degree of genetic heterogeneity and was bred for at least four generations (Chia, Achilli, Festing, & Fisher, 2005; Marmiroli et al, 2017)**.**

Specific Aim 1

The first specific aim was to test the role of cisplatin in development of mechanical allodynia as measured by decreased paw withdrawal threshold to grams of pressure on the hind paw (von Frey filaments). We predicted that cisplatin would significantly decrease paw withdrawal threshold compared to control mice. We also predicted that each strain of mice would develop different degrees of mechanical allodynia as compared to control.

Findings. The results of aim 1 showed that the suggested cisplatin dose produced different degrees of mechanical allodynia in each mouse strain. However, when trying to repeat the results with the strains that developed the most (C57BL/6J) and least (A/J) paw withdrawal responses from baseline, mice from the A/J strain suffered extreme weight loss and had to be euthanized after the first week of treatment.

The results are important because we demonstrated that the same dose of cisplatin led to CIPN development in C57BL/6J mice ($F_{(2,60)} = 44.87$; $p < 0.001$), but not in A/J mice. Both C57BL/6J and A/J are inbred strains of mice, meaning that each C57BL/6J mouse is genetically identical to other C57BL/6J mice and the same is true for the A/J mice, but the C57BL/6J are genetically distinct from A/J mice. The results of nocicfensive behavioral testing therefore imply that the differences in the development of CIPN stem from changes in the genetic background of the mice. Therefore, aim 2 was performed in an attempt to discover novel genes that might explain the difference in withdrawal responses.

Specific Aim 2

The second specific aim was to use both genome-wide expression analysis (microarray) and pathway analysis (Ingenuity Pathway Analysis; IPA) to determine gene expression and compare the relationships between differentially expressed genes in the two strains of mice that represent the highest and lowest responders to behavioral testing (von Frey filaments) before and after cisplatin treatment. We hypothesized that expression differences were present in genes associated with the development of CIPN.

Findings. Using microarray as the method of genome-wide expression analysis in the LH, we found very few gene expression changes within either the C57BL/6J or A/J after treatment, but hundreds of changes between the strains in the untreated state. Further pathway analysis (IPA) led to the identification of differentially expressed genes with a role in the development of neuropathic pain (*BDNF*, *PRKCD*, *KCNQ2*, *CAMKIV, CREBBP, GFAP*, *IL15, GRIA2*, *GRM7*).

We first examined the results of the microarray using multivariate analysis with both strain and treatment as factors and found that treatment had no effect on gene expression. This finding means that the development of CIPN was not the result of cisplatin treatment, but rather was determined by the baseline gene expression in the untreated mouse. Our next analysis only considered strain and found 1311 differentially expressed genes (598 up-regulated and 713 down-regulated) between untreated (naïve) C57BL/6J and A/J mice. Each of these 1311 genes met both the false discovery rate criteria of ≤ 0.10 and the gene expression fold change criterial of ± 1.5.

Using Ingenuity Pathway Analysis (IPA) we identified the top five canonical, or known biological signaling pathways represented by genes in the dataset that were differentially enriched between the C57BL/6J and A/J strains of mice. The top pathways involve the nervous system and have potential implications in development of CIPN; for example, synaptic long-term potentiation, is an increased response to repeated inputs after stimulation and has been thought of as one of the mechanisms underlying the development of central sensitization, of which allodynia is a sign (Baron, Hans, & Dickenson, 2013). CREB signaling in neurons, another top pathway, is implicated in the hyperactivity of dorsal horn neurons after noxious stimulation and is up-regulated in spinal cord injury male rats that have developed neuropathic pain (Crown, Ye, Johnson, Xu, McAdoo, & Hulsebosh, 2006). CREB also has a direct association with increased gene expression in rats exhibiting mechanical allodynia (Crown, E.D., Ye, Z., Johnson, K.M., Xu, G.Y., McAdoo, D.J., Westlund, K.N., Hulsebosch, C.E., 2005).

Each of the pathways was related to neuronal function and could potentially affect the development of CIPN. We next identified eight genes (*PRKCD*, *KCNQ2*, *CAMKIV*, *CREBBP*, *GFAP*, *IL15*, *GRIA2*, *GRM7*) from the pathway analysis that met the criteria for FDR and fold change; and had a documented role in neuropathic pain for further investigation. In addition, a ninth gene, that only met the fold-change criteria, (*BDNF*) was recognized based on its documented role in neuropathic pain. The *CAMKIV* gene was present in four of the top five canonical pathways identified via IPA (CREB signaling in neurons, synaptic long-term potentiation, BMP signaling, and neuropathic pain signaling in dorsal horn neurons) *GRIA2*, *GRM7*, and *PRKCD* were present in three of the top five pathways (CREB signaling in neurons, synaptic long-term potentiation, and neuropathic pain signaling in dorsal horn neurons). In addition, five genes (*CAMKIV*, *CREBBP*, *GRIA2*, *GRM7*, *PRKCD*) were present in two top pathways (CREB signaling in neurons and synaptic long-term potentiation). Other relevant genes from the IPA core analysis were included. *BDNF* and *KCNQ2* were both expressed in the neuropathic pain signaling in dorsal horn neurons canonical pathway. *IL15* was included in inflammatory effects (a biological function). *GFAP* was the top down-regulated gene in the dataset.

The results of aim 2 demonstrated that there are important gene expression differences in the LH related to neuronal functioning and development of neuropathic pain. Many of the genes chosen for qPCR and Western blot analysis were present in more than one canonical pathway. The results of pathway analysis are suggestive that, although separate, the pathways are related via shared genes. Therefore, a change in one gene can lead to changes in more than one biological process.

Specific Aim 3

The third specific aim was to use quantitative real-time polymerase chain reaction (qPCR) to verify gene expression changes from the microarray and Western blot to confirm the presence of protein translation from the differentially expressed genes. We predicted that gene expression changes detected in aim 2 would be verified by both qPCR and Western blot.

Findings. We were able to verify direction of expression (up-regulated or down-regulated) in seven of the genes (*BDNF, KCNQ2, GRM7, GRIA2, CAMKIV, IL15, GFAP*) using qPCR. Use of Western blot was only able to identify protein product for PRKCD and CAMKIV.

We used LH tissue of naïve A/J and C57BL/6J mice that was distinctive from the tissue used in the microarray for qPCR experiments to validate mRNA transcripts from the target genes. Seven of the genes (*IL15*, *CAMKIV*, *GRIA2*, *GRM7*, *KCNQ2*, *GFAP*, and *BDNF*) had gene expression changes in the same direction as the microarray results, but at different magnitudes of fold change. The gene expression differences between the two strains of mice were statistically significant in the *IL15* ($p = 0.0411$), *CAMKIV* ($p = 0.0113$), and *GFAP* ($p = 0.0006$) genes. The remaining two genes (*CREBBP* and *PRKCD*) showed gene expression changes in the opposite direction from the microarray. The results of the qPCR mean that we were able to verify gene expression differences to be in the same direction as the microarray for the seven above listed genes therefore verifying the results of the microarray.

To determine whether the differences in gene expression were also present in the transcribed protein product, Western blot analyses were performed. Analysis of protein lysate from LH tissue of both C57BL/6J and A/J naïve mice identified the targeted protein products for two genes. The protein product of PRKCD was up-regulated 195% in the A/J mice as compared to the C57BL/6J mice, and the protein product of CAMKIV was down-regulated 12% in the A/J as compared to the C57BL/6J mice. What these results mean is that in PRKCD and CAMKIV, the differential expression in the transcriptome led to a difference in protein translation between the strains of mice. Although the results of the Western blots were not congruent with the qPCR. In the qPCR, *CAMKIV* was up-regulated and *PRKCD* was down-regulated in the microarray and up-regulated in qPCR.

Limitations

There were several limitations to this research. After the first week of cisplatin treatment, the A/J cohort of mice had to be euthanized due to > 20% weight loss. We decided not to re-dose and test a new set of animals for a couple reasons. The first reason was that we were able to collect the one-week data. After one week of testing, the difference in CIPN development between the strains of mice was evident. The cisplatin group was significantly different from controls in the C57BL/6J group while there was no significant different between cisplatin-treated and control mice in the A/J mice. Our findings are in agreement with previous reports that have demonstrated that A/J mice appear to be less sensitive to nociceptive stimulation (Mogil, et al. 1999a,b). The second reason was that, from the microarray analysis, we had discovered that cisplatin did not promote gene expression changes, and that the differences in CIPN development were a result of baseline gene expression. Therefore, further qPCR and Western blot testing was performed using naïve, non-treated mice.

A second limitation is related to our choice of array for the microarray. The GeneChip® Mouse Genome 430 2.0 array from Affymetrix (Carlsbad, CA, USA) that we used is constructed using probes from the 3'end of the transcript. This configuration means that the GeneChip® only captured full gene transcripts, and any gene that was alternatively spliced could have been missed. Another drawback is that only known genes are included on the array; therefore, the results of our analysis were limited to the genes and probes on the array chip.

The third limitation concerns the method of protein detection used in our study. We only used Western blot to identify protein in this study. We were able to detect protein with each of our antibodies using protein lysate from whole brain samples, but, due to the size of the lateral hypothalamus in mice, only a small amount of protein was able to be extracted. When considering the abundance of each gene, it may be more appropriate to use a more sensitive protein detection assay, such as enzyme-linked immunosorbent assay (ELISA). Therefore, the lack of protein detection for the seven remaining genes might have been due to the detection method used.

Lastly, this study only used LH tissue from female mice. There is documented evidence that the innervation of the LH is different between female and male rats (Wagner et al., 2016), and we do not know if there is a difference in gene expression in the LH between male and female mice. Further research examining gene expression in male mice is necessary to not only compare gene expression before and after cisplatin treatment, but to also explore gene expression of male mice in comparison to female mice.

Recommendation for Future Research

The results of this preclinical research revealed that the development of CIPN may be in part due to gene expression levels prior to chemotherapy treatment. However, our results are not definitive, and further research in the area is warranted. The results of our preliminary nocicfensive behavioral testing showed different rates of allodynia development between the six strains of mice, but our further investigation only utilized the two strains of mice with the most sensitive (C57BL/6J) and least sensitive (A/J) responses to von Frey filaments. To understand more about the influence of baseline gene expression levels on CIPN development, further work should be performed to analyze the microarray results for the remaining inbred strains of mice. Including these additional strains will increase the heterogeneity of the findings, thus making the results more applicable for translational research.

A second recommendation for further research is perform the same experiment using male mice. As, mentioned above, there is evidence of a sex difference in the LH of rats (Wagner et al., 2016). Having only used female mice for the current study, we are not certain if there is a difference in LH gene expression between the sexes. Although, the final analysis compared naïve mice, it is still important to look at the gene in expression in the LH of male mice before and after treatment. It could be possible that cisplatin treatment promotes gene expression changes in the LH of male mice, but not female. Further research is necessary to determine if there is a distinction in LH gene expression between male and female mice.

A third recommendation is to repeat the study with a different neurotoxic chemotherapeutic to determine if there are gene expression changes attributed to different drugs. The symptom manifestation of CIPN varies based on agent used. For example, CIPN after cisplatin treatment is characterized mainly with sensory impairments, while patients who receive the chemotherapy drug, vincristine also experience motor and autonomic nerve impairments (Park et al., 2013). It is not unreasonable to suggest that the gene expression prolife associated with CIPN after cisplatin treatment is distinct from the gene expression profile associated with CIPN attributed to vincristine.

Lastly, the experiment should be repeated at different time-points. An advantage of using microarray is that it allows for the interrogation of every gene being expressed in a particular tissue at a particular time-point. We did not detect many differentially expressed genes after four weeks of cisplatin treatment, but we do not have the evidence to extrapolate this finding beyond the four-week time-point without further research. It could be that transient gene expression changes occur early after drug induction and the persistence of CIPN is related to early changes that are no longer present at four weeks.

Conclusions

Only a subset of individuals that receive neurotoxic chemotherapy will develop CIPN. The management of CIPN is challenging because not every patient responds to treatment efforts and there are no consistent, reliable, and valid means by which to predict who will develop CIPN. At present, there are no preventative measures and only one approved treatment that is not effective for everyone (Hershmann et al., 2014). Consequently, there is still a need for an evidence-based measure to determine whether an individual is at risk for developing CIPN. A recent review found that, although much effort has been exerted into identifying a genetic profile to identify individuals at risk for CIPN, research results vary widely (Argyriou, Bruna, Genazzani, & Cavaletti, 2017). The majority of these clinical studies are genome-wide association studies using a candidate gene approach. This differs from our pre-clinical study because rather than limiting ourselves to certain candidate genes, we looked at the entire LH transcriptome. This allowed us to examine every gene being expressed in the LH at the time of tissue harvest and not a finite list of predetermined genes, thus permitting the discovery of novel gene targets. In terms of platinum-related CIPN, clinical studies are targeted more towards the genes that encode proteins for ion channel activity, neuronal development and function, as well as apoptosis, and oxidative stress (Argyriou et al., 2017). The genes examined in our study are active in these functions but rather than examining how the genes are changed by chemotherapy, our study found that it may be the expression levels of these genes at baseline that predicts who develops CIPN. With the current focus on the practice of precision medicine, this could be a prime example of a condition that is individualized to each patient. So that, perhaps, in the future, prior to receiving neurotoxic chemotherapy, each patient will undergo a genetic testing of baseline expression of a certain gene to determine whether or not they will develop CIPN.

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APPENDIX

Appendix

Table 4-4. Pain-associated genes differentially expressed in lateral hypothalamus tissue of naïve AJ mice as compared to naïve C57BL/6 mice.

Affymetrix Probe set ID	Entrez ID	Gene symbol	Gene name	Fold change	False Discovery Rate	F-statistic	Summary
1422168_a_at	12064	*BDNF*	brain derived neurotrophic factor	-1.690615	0.300882	2.1750	Location: Chr 2. The *BDNF* gene encodes for a protein important in growth and development of the nervous system (Huang & Reichardt, 2001). Although the *BDNF* gene expression was not found to be significant according to our *a priori* criteria, *BDNF* presence has been shown to be a factor in the depolarizing neuronal anion gradient in the spinal cord dorsal horn that underlies neuropathic pain development in rats (Coull et al., 2005). *BDNF* was also part of the "Neuropathic Pain Signaling in Dorsal Horn Neurons" canonical signaling pathway in Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com). The development of allodynia, as seen in cisplatin treated mice of the current experiment is a sign of neuropathic pain.
1422847_a_at	18753	*PRKCD*	Protein kinase C, delta	-2.937064	0.038828	5.9441	Location: Chr 14. The *PRKCD* gene encodes for a protein that phosphorylates target proteins in cellular signaling pathways. PRKCD protein in primary afferent sensory neurons is activated by paclitaxel in a mouse model of chemotherapy-induced peripheral neuropathy (He and Wang, 2015). It has also been shown that PRKCD protein inhibitors enhance the

							anti-cancer effect of cisplatin in mice (Zhang, Xu, & Dong, 2017) giving PRKCD a negative role in cisplatin therapy. The *PRKCD* gene was present in three of the top five canonical signaling pathways differentially enriched in the dataset.
1420800_a_at	16536	*KCNQ2*	potassium voltage-gated channel, subfamily Q, member 2	-2.142803	0.001304	15.1514	Location: Chr 2. The *KCNQ2* gene encodes for a potassium voltage-gated ion channel. The KCNQ2 protein channel transmits a particular type of current called the M-current. The M-current is a slowly activating and deactivating potassium channel that plays a critical role in the regulation of neuronal excitability. Activation of the KCNQ2 channel hyperpolarizes neuronal membranes, therefore inhibiting hyperexcitability (Wu et al., 2013). Hyperexcitability of the membrane is part of the underlying mechanism of neuropathic pain, of which allodynia is a sign. It has been shown that *KCNQ2* gene transcription is down-regulated after neuropathic injury in male rats (Rose et al., 2011), which does not correlate with results of the current study that demonstrated down-regulation in allodynia-resistant A/J mice. The *KCNQ2* gene is also part of the IPA "Neuropathic Pain Signaling in Dorsal Horn Neurons" canonical signaling pathway.
1426167_a_at	12326	*CAMKIV*	calcium/ calmodulin-dependent protein kinase IV	1.782235	0.012568	8.5095	Location: Chr 18. The *CAMKIV* gene encodes for a kinase in the serine/threonine protein kinase family, and the calcium/calmodulin-dependent protein kinase subfamily. The CAMKIV protein phosphorylates the

							cyclic AMP response element-binding protein (CREB). An increase in phosphorylated CREB has been shown in the spinal cord after chronic constriction injury (CCI) of the sciatic nerve (a model of neuropathic pain) in male rats (Miletic, Pankratz, & Miletic, 2002), implicating an increase in CAMKIV protein with neuropathic pain. Gene expression level may be tissue dependent, as supraspinal presence of CAMKIV increased antinociception in male mice (Jackson & Damaj, 2013). The *CAMKIV* gene was included in four of the top five canonical signaling pathways that were differentially enriched in the dataset.
1459804_at	12914	*CREBBP*	CREB binding protein	1.629148	0.000859	16.7278	Location: Chr 16. The *CREBBP* gene is ubiquitously expressed and is involved in the coactivation of many transcription factors. Increased immunoreactivity of the CREBBP protein is reported in the spinal cord dorsal horn of male rats following CCI surgery (Zhu et al., 2013). This is consistent with studies that show that inhibiting CREBBP with curcumin post CCI (Zhu et al., 2014) or knockdown of *CREBBP* with an antisense oligodeoxynucleotide in a model of antiretroviral-induced neuropathy (Iida et al., 2016) reduce allodynia in the spinal cord dorsal horn of male rats. *CREBBP* is also part of a gene set associated with gene up-regulation in the DRG in rat models of neuropathic pain (Maratou et al., 2009). The *CREBBP* gene is present in two of the top five differentially

							enriched canonical signaling pathways from the dataset.
1440142_s_at	14580	*GFAP*	Glial fibrillary acidic protein	-9.784597	0.000322	20.7406	Location: Chr 11. The *GFAP* gene encodes for a cytoskeletal component of cells used as a marker to distinguish astrocytes from other glial cells during development. Treatment with oxaliplatin (Robinson, Zhang, & Dougherty, 2014), bortezomib (Robinson et al., 2014, Robinson & Doughetry, 2015), and paclitaxel (Zhang et al., 2012) result in increased expression of GFAP protein in male rats. Pathway analysis of the current dataset showed the *GFAP* gene as one of the top 10 down-regulated genes in the dataset.
1418219_at	16168	*IL15*	Interleukin 15	-1.521174	0.006391	10.2072	Location: Chr 8. The *IL-15* gene encodes for a T-cell cytokine that regulates immune cell activation (Gomez-Nicola et al., 2008; Sun et al., 2013). The IL-15 protein is increased in the spinal cord of male rats at early time points (6 h to 5 days) after neuropathic pain development via CCI of the left sciatic nerve (Gomez-Nicola, et al., 2008). Clinical work has shown serum levels of the IL15 protein are significantly higher in osteoarthritis (OA) patients reporting more pain (Sun et al., 2013). Both of these studies show increase IL-15 with the development of pain.
1453098_at	14800	*GRIA2*	glutamate receptor, ionotropic, AMPA2 (alpha 2)	1.657999	0.004147	11.4177	Location: Chr 3. The *GRIA2* gene encodes a subunit of the ionotropic GluR2 protein. GluR2 is important for alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)

							functions including calcium permeability and synaptic plasticity (Issac, Ashby, & McBain, 2007;Liu et al., 2015). There is evidence that GluR2 is involved in neuropathic pain development at both the spinal (Chen, Zhou, Byun, & Pan, 2013) and supraspinal (Ho, Cheng, & Chiou, 2013; Liu et al., 2015) levels. Nerve injury via spinal nerve ligation leads to internalization of GluR2 in the spinal cord and increased pain hypersensitivity in male Sprague Dawley rats (Chen et al., 2013). Supraspinally, nerve injury from CCI also leads to GluR2 internalization in the periaqueductal gray, a midbrain structure where descending pain inhibition can be initiated (Ho, Cheng, & Chiou, 2013; Liu et al., 2015). The GRIA2 gene is present in two of the top five differentially enriched canonical signaling pathways from the dataset.
1459532_at	108073	*GRM7*	glutamate receptor, metabotropic 7	1.806628	1.42E-05	40.0795	Location: Chr 6. The *GRM7* gene encodes the metabotropic GluR7 protein that is expressed on both neurons and glial cells throughout the nervous system (Chiechio, 2016). The mGluR7 protein receptor can be located on both glutaminergic and GABAminergic neurons. Supraspinally, GluR7 receptors are preferentially located on glutaminerigc synapses (Bradley et al., 1996; Shigemoto et al., 1997; Marabese et al., 2007). Presynaptically located Glur7 provides negative feedback for the release of glutamate and other neurotransmitters and is implicated in the development of central sensitization (Chiechio, 2016). Allodynia is a sign of central sensitization, a physiological

	change that takes place with neuropathic pain development. Stimulation of GluR7 in supraspinal areas such as the PAG and amygdala increase pain while neuropathic pain is decreased when GluR7 is blocked in these same areas (Chiechio, 2016). The *GRM7* gene is differentially regulated in three of the top five canonical pathways.

Notes. *p < .05. All gene summaries from Gene cards: http://www.genecards.org. Other references are delineated in the references section.